MASTER THESIS IN CANCER BIOLOGY

Irradiation Regulated Secretome of ADAM17

A Focus on Intra- and Intercellular Communication dependent on ADAM17

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STATEMENT OF AUTHORSHIP

I declare that I have used no other sources and aids than those indicated. All passages quoted from publications or paraphrased from these sources are indicated as such, i.e. cited and/or attributed. This thesis was not submitted in any other form for another degree or diploma at any university or other institution of tertiary education.

Zurich, August 2018

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ABBREVIATIONS

ADAM17 / A17	A Disintegrin and Metalloproteinase 17
ALCAM	Activated Leukocyte Cell Adhesion Molecule
CAF	Cancer-associated Fibroblast
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DNA	Deoxyribonucleic acid
Dox	Doxycycline
DSB	Double Strand Break
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immunosorbent Assay
EMT	Epithelial-to-mesenchymal transition
FCS	Fetal Calf Serum
G.O. I	Gene of interest
GFP	Green Fluorescent Protein
Gy	Gray
h	Hour
HEK293	Human Embryonic Kidney Cells
HR	Homologous Recombination
IR	Ionizing Radiation
LB	lysogeny broth
mAB	Monoclonal Antibody
MET	Mesenchymal-to-epithelial transition
MMP	Matrix Metalloprotease
mut	Mutant
NHEJ	Non-homologous End Joining
NSCLC	Non-small cell lung cancer
P/S	Penicillin-Streptomycin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PET	Polyethylene terephthalate
PGK	Phosphoglycerate kinase 1
Puro	Puromycin
PVDF	Polyvinylidene fluoride
RFP	Red Fluorescent Protein
RIBE	Radiation Induced Bystander Effect
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPM	Rounds per Minute
RT	Radiotherapy
RTK	Receptor Tyrosine Kinase
SD	Standard Deviation

SDS	Sodium dodecyl sulfate
Secretome	Serum proteome
SEM	Standard Error of the mean
shRNA	Short-hairpin RNA
SSB	Single Strand Break
STR	Short tandem repeat
TACE	Tumor necrosis factor α converting enzyme
TME	Tumor Microenvironment
wt	Wildtype

SUMMARY

One of the biggest and most important challenges of modern science is to defeat cancer. Decades of research have significantly improved the therapeutic outcome, yet have failed to satisfactorily control all of the different types of cancer. The reasons are most likely due to the heterogeneity of the development of cancer: mutations, epigenetic changes, abnormalities and defects in multiple different genes and proteins can lead to aberrant cell behavior, that can ultimately result in the disease called “cancer”. Radiotherapy is a standard treatment strategy for cancer patients, applied alone or in combination with other treatment regimes, and mostly aims to kill tumor cells by DNA damage. Interestingly, tumor cells exposed to ionizing radiation release several factors into the tumor microenvironment where they influence signaling cascades in an auto- and/or paracrine fashion. Radiotherapy kills a clear majority of tumor cells, but also induces a multilayered stress response that may interfere with optimal treatment outcome.

In foresight to the development of novel drug targets that can be used to improve radiotherapy, my thesis focuses on the intra- and intercellular signaling pathways orchestrated by the metalloproteinase ADAM17. ADAM17 is localized on the outer side of the plasma membrane and cleaves multiple factors involved in tumor progression and inflammation. In several cancer types, ADAM17 expression is increased compared to healthy tissue and correlates with poor prognosis. Our previous studies also implicated ADAM17’s role in radiosensitizing cells towards ionizing radiation.

In a first part, I aimed to identify the role of a shRNA-mediated downregulation of ADAM17 in two different NSCLC cell lines (A549 and H358) in response to IR. Downregulation of the protein levels concomitantly resulted in decreased enzyme activity and subsequent ligand shedding. Additionally, ADAM17-depleted cells showed decreased proliferative activity and clonogenic survival in a dose-dependent way. Based on this evidence, we conclude that ADAM17 is involved in mechanisms influencing efficacy of ionizing radiation.

In a second part, we investigated ADAM17’s effect on intra- and intercellular signaling affecting migration. Astonishingly, migration was increased towards a secretome full of ADAM17-cleaved factors as compared to an ADAM17-cleaved factor scarce secretome. These results indicate an involvement of ADAM17-cleaved factors in intra- and intercellular communication affecting migration.

In conclusion, the thesis underlines the undeniable involvement of ADAM17 in orchestrating radioresistance. Additionally, it helps to further explain the intra- and intercellular regulatory mechanisms facilitated by ADAM17. Altogether, the work of this thesis supports the rational of combining radiotherapy with a potent ADAM17 inhibitor to improve treatment outcome.

ZUSAMMENFASSUNG

Die Bekämpfung von Krebs zählt heutzutage zu den grössten wissenschaftlichen Herausforderungen. Jahrzehntelange Forschung hat die Krebsbehandlung signifikant verbessert, allerdings können bis heute nicht alle Tumore in einer befriedigenden Weise kontrolliert werden. Die Gründe dafür liegen mit grösster Wahrscheinlichkeit in der enormen Vielfältigkeit der Entstehung von Tumoren. Mutationen, Abnormalitäten und Defekte in vielen verschiedenen Genen und Proteinen können zu anormalem Zellverhalten führen, welche zum Krankheitsbild «Krebs» führen können. Die Radiotherapie, angewendet alleine oder in Kombination mit anderen Behandlungsstrategien, zählt zu den Standardbehandlungen von Krebspatienten und hat zum Ziel Tumorzellen, mittels DNS Schädigungen, zu töten. Interessanterweise sondern bestrahlte Zellen viele Faktoren ins Tumormikromilieu ab, wo sie Signalkaskaden in auto- und parakriner Weise beeinflussen können. Durch die Radiotherapie werden folglich eine grosse Anzahl von Tumorzellen getötet, allerdings induziert sie ebenfalls eine vielschichtige Stressantwort, die mit dem optimalen Behandlungsresultat interferieren kann.

Im Hinblick auf die Entwicklung neuer Angriffspunkten von Medikamenten, welche der Verbesserung der Radiotherapie dienen, fokussiert sich diese Masterarbeit auf die intra- und interzellulären Signalwege, die von der Metalloproteinase ADAM17 beeinflusst werden. ADAM17 befindet sich an der äusseren Seite der Plasmamembran und ist für das Schneiden vieler Faktoren, welche in die Tumorprogression und Entzündung involviert sind, verantwortlich. In zahlreichen Krebsarten ist die ADAM17 Expression erhöht, was wiederum mit einer schlechten Prognose korreliert. Ebenfalls konnten verschiedene vorhergehende Studien darlegen, dass das ADAM17 Protein eine Rolle in der Radiosensitivität hat.

Ziel dieser Masterarbeit ist, den Effekt von einer durch shRNA-generieter ADAM17 Herunterregulierung in zwei verschiedenen nicht-kleinzelligen Lungenkrebs Zelllinien zu analysieren, wobei der Fokus besonders auf deren Verhalten nach Bestrahlung gelegt wurde. Die resultierende Protein-Herunterregulierung führte gleichzeitig zu einer verringerten Enzymaktivität und darauffolgender reduzierter Liganden-Absonderung. Zusätzlich haben ADAM17-defiziente Zellen eine Strahlendosis abhängige, reduzierte Wachstumsrate und klonogenes Überleben. Daraus kann geschlossen werden, dass das ADAM17 Protein in Mechanismen involviert ist, welche die Effizienz von ionisierender Bestrahlung beeinflussen.

Im zweiten Teil dieser Arbeit wurde die Beteiligung von ADAM17 in intra- und interzellulärer Kommunikation, in Bezug auf Migration, analysiert. Erstaunlicherweise ist die Migration zu einem Sekretom voll mit ADAM17-Liganden erhöht gegenüber der Migration zu einem Sekretom mit spärlichen Mengen von ADAM17-Liganden. Diese Resultate deuten mindestens bezüglich der Migration auf eine starke Beteiligung von ADAM17 in der intra- und interzellulären Kommunikation hin.

Summa summarum unterstreicht diese Arbeit die unbestreitbare Mitwirkung von ADAM17 in zellulärer Radioresistenz. Zusätzlich öffnet sie die Möglichkeit zur Identifizierung der intra- und interzellulären Regulationsmechanismen, welche durch

das ADAM17 Protein ermöglicht werden. Schliesslich unterstützt diese Arbeit ebenfalls die Evidenz für die Kombination der Radiotherapie mit einem potenten ADAM17 Inhibitor, um bessere Behandlungsresultate zu erzielen

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1 INTRODUCTION

1.1 HALLMARKS OF CANCER

Cancer includes all different types of neoplastic growth, from benign cell accumulations to highly aggressive and invasive tumor sites. A tumor (Latin tumere: “to swell”) describes “a swelling of a part of the body, generally without inflammation, caused by an abnormal growth of tissue, whether benign or malignant” (oxford dictionary [1]). Each tumor is shaped in a dynamic and unique way, making it a challenge to determine the genetic and epigenetic alterations underlining the cause, maintenance and spread of malignant cells. Features allowing cells to become malignant cancer cells include traits that enable them to survive, proliferate and disseminate. Robert Weinberg and Douglas Hanahan described these different characteristics in 2000 in “The Hallmarks of Cancer”, concentrating the complexity of cancer into six main characteristics. Later, these hallmarks were completed by four more characteristics, resulting in now ten hallmarks of cancer (Figure 1). [2, 3]

The ability of cancer cells to sustain chronic proliferation is a fundamental trait. In normal tissue, the production and release of growth promoting signals are carefully controlled, ensuring a homeostasis of cell number, maintenance of normal tissue architecture and function. Cancer cells, in contrast, can acquire sustained proliferative signaling by several alternative ways: producing growth factor ligands by themselves, stimulating normal cells to supply them with various growth factors, elevating the levels of receptor proteins thereby rendering the cells hyper-responsive to the limiting amounts of growth factor ligands or creating structural alterations in the receptor molecules to mediate ligand-independent firing. Defects in feedback mechanisms which ensure homeostatic regulation of the flux of signals can enhance proliferative signaling.

Upregulating growth factor ligands is not enough, as cancer cells must also circumvent powerful signals that negatively regulate cell proliferation. Such signals often depend on tumor suppressor genes that govern the decisions of cells to proliferate or to activate senescence or apoptosis. Programmed cell death by apoptosis is a natural blockage to cancer development. Apoptosis can be attenuated in tumors that thrive in progressing to advanced states of malignancy, most commonly by the loss of the TP53 tumor suppressor function.

Unlimited growth seems to be restricted by the limited amount of successive cell growth-and-division cycles normal cells can pass through. In each cell cycle, the length of the telomeres (repetitive DNA at the end of chromosomes) is shortened and when the telomeres have lost their protective function, they will trigger crisis to prevent chromosomes from end-to-end fusions. Telomeres are centrally involved in the process of unlimited proliferation. Cancer cells express the specialized DNA polymerase telomerase which subsequently adds telomere repeat elements to the existing once, hence the chromosome is never threated from too short telomeres.[4] Telomerase activity provides resistance to senescence and crisis/apoptosis.

To sustain the growing tumor mass with oxygen and nutrients, the “angiogenic switch”

is activated and stays on throughout tumor development to continually mount new vessels into the growing tumor.[5] The neovascularization is controlled by a complex signaling network involving the cancer cells and the stromal microenvironment.
The stromal microenvironment is also an important mediator of invasion and metastasis. Invasion and metastasis are multistep processes marked by the downregulation of cytostatic factors and upregulation of molecules associated with migration. Due to the near impossible detection of metastasis and the possible spread to all kind of organs, invasion and metastasis are a great threat resulting in the most often cause of death. [3]
But what allows the cancer cells to survive, proliferate and disseminate? Hanahan and Weinberg described, additionally to the six Hallmarks of cancer, the four enabling characteristics. The most prominent enabling characteristic is genomic instability in cancer cells, which includes rare genetic changes orchestrating hallmark capabilities. A second enabling characteristic involves the inflammatory state of tumor lesions. Inflammation supplies bioactive molecules (e.g. growth factors, survival factors, proangiogenic factors) to the tumor microenvironment. [6] The third describes the cancer cells ability to cope with the increased need of energy of the forming tumor by reprogramming the cellular energy metabolism. Most notably, cancer cells limit their energy metabolism largely to glycolysis even in the presence of oxygen. This reprogramming was first described by Otto Warburg and is thus called the “Warburg

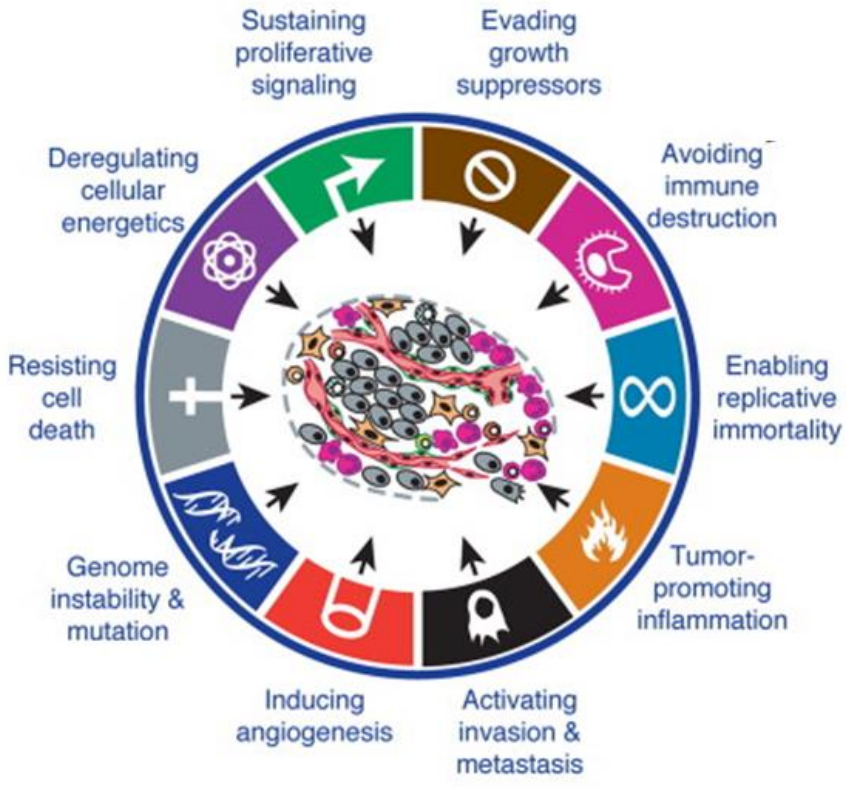

Figure 1. Ten Hallmarks of Cancer by Weinberg and Hanahan (2011)
Weinberg and Hanahan described the 10 Hallmarks of Cancer.
Figure adapted from [2].

Effect". [7] Last but not least, tumors manage to avoid detection by the immune system or limit the extent of killing by immune cells, and hence can continue to grow and spread.

1.1.1 Tumor Microenvironment/Metastasis

Metastases arise when cancer cells migrate and invade into distal healthy tissue. To acquire this migratory phenotype, cancer cells increase the expression of genes required for cell motility, which respond to cues from the microenvironment, ultimately triggering invasion.

The tumor microenvironment (TME) consists of cancer associated fibroblasts (CAF), endothelial cells, inflammatory cells, extracellular matrix (ECM) and diffusible molecules (growth factors, cytokines). Cancer cells are in constant interaction with the TME and this crosstalk can promote tumor progression by conferring cancer cells with the ability to proliferate, migrate, invade and metastasize (Figure 2). Specialized intercellular junctional proteins maintain epithelial cell contact with neighboring cells, allowing the cancer cells to communicate with each other, either directly by cell-to-cell contact or indirectly in a paracrine fashion. The release of various inflammatory cytokines, growth factors and proteases forms a metastasis-permissive microenvironment. [8, 9]

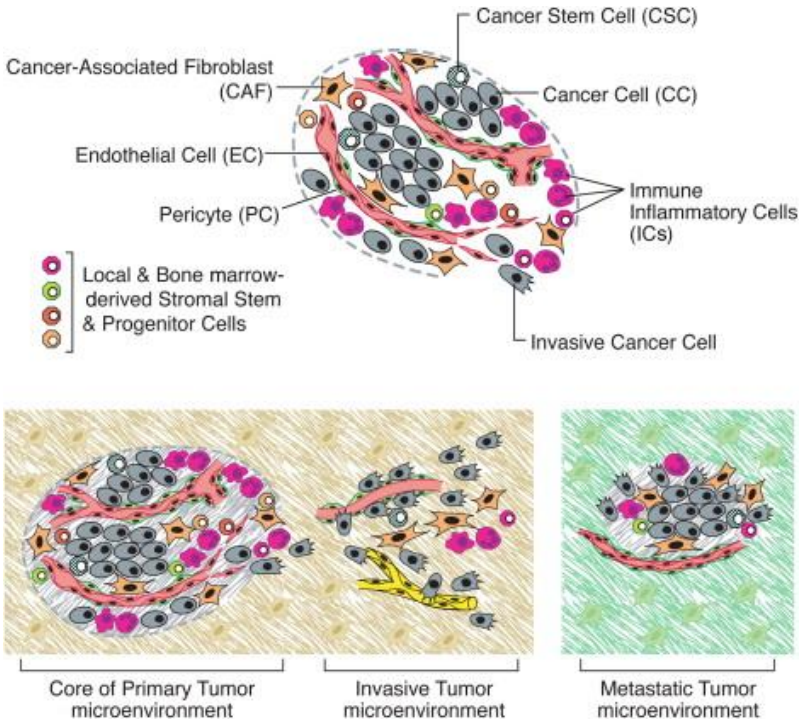

Figure 2: Metastasis is a multistep process relying on the communication between cancer cells and the tumor microenvironment

The tumor microenvironment consists of many different cell types, collectively enabling tumor growth and progression. It evolves throughout cancer progression thereby enabling migration, invasion and metastatic growth. [2]

The epithelial-to-mesenchymal-transition (EMT) marks the beginning of the invasion process (of epithelial-derived cancers = carcinomas). First, cancer cells lose their cell-cell adhesion structures, change their polarity and organization of their cytoskeleton and become isolated and motile.[10] At distant tissues, the cells can extravasate into the parenchyma, undergoing the mesenchymal to epithelial transition (MET) and form micrometastases. If the local microenvironment permits it, such micrometastases can colonize, forming metastases. Until now, it is not known whether carcinoma cells acquire the capability to invade by activation of parts of the EMT program, or whether alternative regulatory programs, such as a crosstalk between cancer cells and stromal cells, can also enable invasion capability.
The complex signaling interactions between cancer cells and the surrounding nonmalignant stroma evolve throughout the multistep tumor development and represent one of the major challenges in cancer research.

1.2 Treatment Strategies for Cancer

With increased understanding of the molecular mechanisms and characteristics of cancer, the focus of treatment has shifted from relatively general cytotoxic agents to selective, mechanism-orientated and personalized therapeutics. Prior to treatment and to personalize the treatment strategy, the stage, location within the body, the grade, the genetic background, the metastatic status and the general health status of the patient are assessed.
The general treatment strategies are surgery, radiotherapy, chemotherapy, molecular target therapy, immunotherapy and the combination of those.

1.2.1 Radiotherapy

Radiotherapy uses ionizing radiation (IR) to kill cancer cells, mainly by causing DNA damage that eventually leads to cell death.
Ionizing radiation is defined by the localized release of large amounts of energy. It can be classified as directly or indirectly ionizing. Protons and Electrons are directly ionizing: if the individual particles have sufficient amount of kinetic energy, they can disrupt the atomic structure of the absorber (in the cell the absorber may be proteins, lipids and the DNA molecule). This generates chemical and biological changes. Electromagnetic radiations (x- and γ-rays) are indirectly ionizing, meaning they do not produce chemical and biological damage themselves. X- and γ-rays transmit their energy when they pass through the absorber which subsequently produces fast-moving, charged particles which then can produce damage.
Additionally, the effects of x- or γ-rays can be subdivided into direct or indirect (Figure 3). The direct action of x- or γ-rays lays in the absorbance in biological material, directly interacting with the critical targets of the cells. The target itself will be ionized or excited and subsequently initiate the chain of events leading to biological changes. Alternatively, ionizing radiation produces free radicals by interacting with atoms or molecules in the cell (mainly water). When a photon of x- or γ-rays interacts with a water molecule, the water molecule becomes ionized, becoming an ion radical ($H_2O\bullet^+$) that is charged and has an unpaired electron. The ion radical reacts with another water molecule, forming the highly reactive hydroxyl radical OH• which can diffuse to a critical

target of the cell, causing biological damage. [11]
The most important biological impairment of IR is DNA damage. Single-Strand Breaks (SSB) occur prevalently but are of little biological consequence because they can be repaired efficiently by repair mechanisms of the cell by using the other strand as template. A radiation induced DNA Double Strand Break (DSB) causes the disruption of chromatin into two pieces and yields a greater threat concerning cell killing, carcinogenesis and mutation. Cancer cells frequently have mutations in genes controlling the DNA damage response. This and their highly proliferative character make them especially susceptible to DNA damage induced by IR. [12]

Radiation Therapy (RT) is used in ~50% of patients with solid tumors, either as primary strategy to target the tumor, as palliative treatment or in combination with other treatment modalities (surgery, chemotherapy or immunotherapy). It can be administered by an external beam from outside the body which points the high-energy rays to the location of the tumor (most common approach) or it can be administered as internal radiation (or Brachytherapy) from inside the body. Radiation is given in fractions, giving the normal, healthy cells time to repair the damage caused to the DNA and therefore reducing normal tissue toxicities to the body.[13]

1.2.1.1 5 R's of Radiotherapy

In 1975, Rod Withers published a paper describing the "The 4 R's of Radiotherapy". This short list contained the mechanisms which are important in determining the response of a biological tissue to multiple doses of radiation: Repair, Reassortment, Repopulation and Reoxygenation. These 4 R's are still of great importance, though they have been completed with a fifth R, Radiosensitivity. [14] In optimizing these key biological parameters, local tumor control can be improved, and normal tissue toxicity minimized.
The first R, Repair, stands for the efficient repair of lesions such as DNA SSBs and

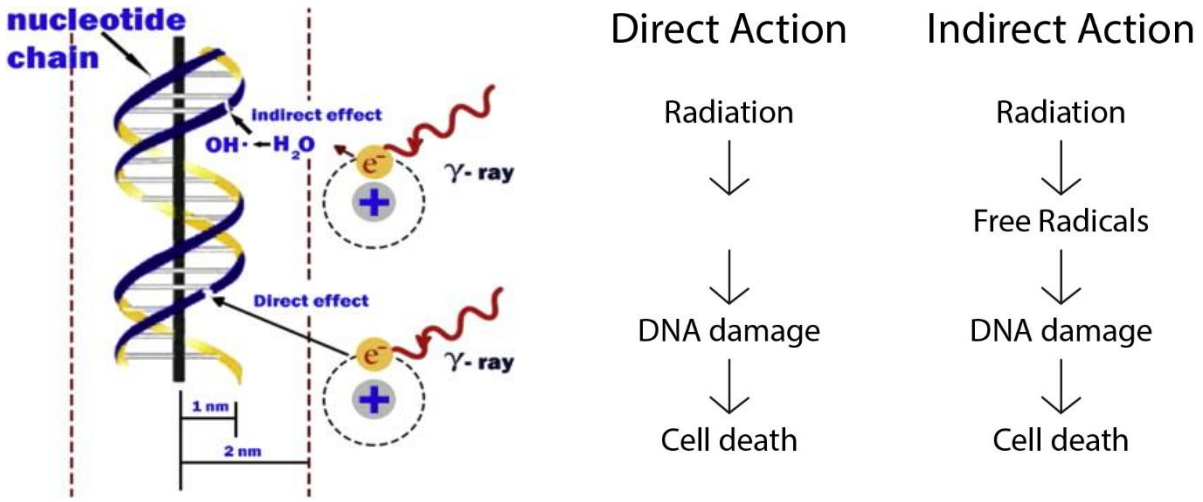

Figure 3. Direct and Indirect Actions of Ionizing Radiation
Ionizing radiation (X and γ-rays) can act directly on the DNA or indirectly by producing free radicals. Figure adapted from [11]

DSBs. The radiation induced, sublethal damage can only be repaired in cells with intact DNA repair mechanisms. By administering the radiation dose in fractions, normal cells have enough time to repair the damaged DNA whereas cancer cell, with mostly mutated repair mechanisms, cannot. Persistent DNA DSBs can lead to mitotic catastrophe, and eventual cell death.

The second R, Redistribution, stands for the fact that radiation sensitivity varies among the different cell cycle stages. Cells in M and late G2 phase are most sensitive to radiation whereas cells in late S phase are more resistant. This pattern of radiosensitivity correlates with the different mechanism of DNA repair active in different stages of the cell cycle. During late S phase, DNA DSBs are repaired by Homologous Recombination (HR) which is a high-fidelity repair mechanism using the homologous strand of the sister chromatid as a template. When no sister chromatid is available, for instance in M and G2 phase, Non-Homologous End Joining (NHEJ) is the primary repair mechanism. NHEJ is error prone, leading to chromosomal aberrations and accumulation of these aberrations can result in cell death. By having a highly proliferative character, cancer cells constantly go through the different phases of the cell cycle, eventually being in a sensitive phase when a fraction of radiation is applied.

The third R, Repopulation, stands for the radiation-induced accelerated repopulation of tissue as cells try to fill the void created by dying cells.

The fourth R, Reoxygenation, describes the benefit of letting parts of the tumor be re-oxygenated between the fractions. Tumor cells grow at such a high rate without any hindrance, eventually coming to a point where not all cells of the tumor mass have access to oxygen and nutrient delivering blood vessels. Cells without sufficient oxygen do not cycle through the cell cycle stages, residing in the radiation-insensitive phase G1. By fractionating the giving dose, tumor cells at the edge are killed, allowing oxygen to diffuse to the previously blocked inner tumor cells, pushing them through the cell cycle and putting them eventually in a radiation-sensitive phase.

The fifth R, Radiosensitivity, states that apart from Repair, Redistribution, Reoxygenation and Repopulation, the intrinsic radiosensitivity differs between different cell types. More sensitive cell types include stem cells, sperm and egg cells, intestinal cells and blood cells (virtually all actively dividing cells) and more resistant cells types include cells that do not divide, for example neurons or brain cells. [15]

1.2.1.2 Unfavorable Effects of Radiation

The ultimate goal of radiation therapy is to kill target cells, mostly by means of DNA damage that is beyond the repair capacity of cancer cells. Unfortunately, also non-irradiated cells show various biological effects of IR, a phenomenon described as the radiation-induced bystander effect (RIBE).[16] RIBE is mediated by direct cell-cell contact (gap-junction mediated intercellular signaling) or by a range of soluble signaling molecules (e.g. TGF-β, TNF-α, IL-6) dispersing between distanced cells (Paracrine intercellular signaling).

Several factors released upon RT (cytokines, growth factors, ROS), activate receptor-mediated pathways and create a positively regulated loop capable of maintaining a permanent signaling between cells and the tumor microenvironment. This highly active

signaling network can create pathological conditions favorable for tumor invasiveness and cancer progression. [17-19]

1.2.2 Chemotherapy

Chemotherapy consists of drugs mainly interfering with the cells ability to divide properly. Chemotherapeutic agents can be divided into several subgroups: Alkylating Agents are cell-cycle unspecific and act directly on the DNA by causing DNA strand breaks. These result in abnormal base pairing, inhibition of cell division and eventual cell death. [20] Derived from certain types of plants, Plant Alkaloids are cell cycle specific by primarily acting during M phase, where they inhibit the formation of spindles and therefore interfere with the correct chromosome segregation. Antimetabolites are very similar to normal component of the cells and they compete with these normal components for the active site of an essential enzyme. Through that, they can impede for example the DNA (makes them cell cycle specific) and render the cell unable to divide. Another agent, also cell cycle specific, is the topoisomerase inhibitor which inhibits the DNA detangling enzyme topoisomerase, therefore repressing replication.
Chemotherapeutic agents are not cancer type specific, but target in general the proliferative subset of cells in the body (Skin cells, hair cells, cells of the intestinal lining), leading to treatment-induced off-targets toxicities. Normally, chemotherapy is administered as a combination of different types of cytotoxic agents.[21]

1.2.3 Surgery

By practical thinking, a patient with a non-hematological cancer can be cured by removing the malignant cells by surgery. Indeed, many patients undergo surgery to excise the tumor, alone or with the whole organ affected. Unfortunately, tumors, also small ones, can spread to other sites of the body, forming metastases or the remaining, even so little, tumor cells can regrow to form another, maybe even more aggressive, tumor. This is the reason why surgery is often accompanied by other treatment strategies such as chemotherapy or radiation therapy.
Being undeniably one of the most successful single modality, surgery has evolved over the years, also thanks to the rapid technical evolution, resulting in robots that can assist during the surgery (e.g. da Vinci Prostatectomy) and an extinction of surgery as a cancer treatment modality is very unlikely. [22]

1.2.4 Molecular Targeted Therapy

With the remarkable advances in the understanding of the molecular mechanisms underlying malignant progression of cancer, molecular targeted cancer therapy led to many clinical successes. Unfortunately, molecular targeted therapies are faced with many problems such as development of drug resistance, marginal response rate, and short-lived responses followed by disease progression.
Molecular targeted therapy usually blocks the growth and spread of cancer by interfering with distinct molecules responsible for cancer progression (e.g. tyrosine kinase). In contrast to chemotherapy agents that interfere with standard cell mechanisms and molecules, targeted therapies work on molecular abnormalities specific for a cancer type and is consequently less harmful for non-malignant cells.
Growth factor receptors and non-receptor signaling molecules represent the largest class of drivers for cancer cell development and agents targeting exactly such

molecules showed the initial success of targeted therapy, namely with trastuzumab targeting the HER2-RTK and imatinib targeting the non-receptor tyrosine kinase Bcr-ABL. The most recent exciting success with molecular targeted therapy was achieved with the blockade of immune checkpoint molecules (CTLA-4, PD1 and PD-L1) and has led to explicit discussions about revisiting cancer immunotherapy.[23]

1.3 NON-SMALL-CELL-LUNG-CANCER

Lung Cancer is responsible, together with colorectal and prostate (men) or breast (woman), for the most commonly diagnosed cancer. In terms of cancer-related death, lung cancer climbs to the top of that list, being responsible for 27% of cancer-related deaths. [24] More than 85% of the newly diagnosed cases are classified as non-small-cell lung cancer (NSCLC) for which the predicted 5-year survival rate is only at 15.9% - a number that has unfortunately only marginally improved during the last few decades.[25]

NSCLS are subcategorized into adenocarcinoma, squamous cell carcinoma (sqCC) and large cell carcinoma. SqCC is a malignant epithelial tumor reflecting keratinization and/or intercellular bridges. Over 90% of SqCC occur in cigarette smokers. adenocarcinoma is a malignant epithelia tumor with glandular differentiation or mucin production and has surpassed SqCC as the most common histologic subtype of lung cancer. Most cases of Adenocarcinoma are seen in smokers, it however develops more frequently than any other type of lung cancer in non-smokers. Large cell carcinoma is an undifferentiated NSCLC lacking the cytologic and architectural features of small cell carcinoma and glandular or squamous differentiation.[26]

The treatment of NSCLC has evolved over the past decade and early diagnosis and surgical treatment are important for optimal patient outcome. However, the majority of patients are diagnosed only at later, progressed stages and require multimodality therapy. The increased understanding of the molecular heterogeneity underlying cancer initiation and progression, as well as advances in standard of care, significant improvement in the management of patients with advanced stages of lung cancer have been made.[27] Nonetheless, the mortality rate is still very high. Pursuing further research on characterizing NSCLC and developing novel treatment strategies is therefore of utmost importance. [28]

1.4 ADAM17

The surface-expressed disintegrin and metalloproteinase ADAM17 (also known as tumor necrosis factor α-converting enzyme, TACE) is found in most tissues, is constitutively expressed in various cells and plays important roles in divers physiological and pathophysiological processes. [29] It processes single-spanning membrane proteins such as cytokines, growth factors, receptors and chemokines. Up until now, over 80 substrates have been identified, most of which are implicated in cancer and inflammation. ADAM17 has become an attractive target for therapeutic intervention because it has been discovered that, despite its broad substrate profile, it is typically further activated in response to stimuli that drive disease states, for example tumor progression, tumor-induced angiogenesis and hypoxia-induced tumor cell

invasiveness. [30] The study of the shedding events orchestrated by ADAM17 proposed novel mechanisms of resistance to popular cancer therapies. [31]

1.4.1 Protein Structure

ADAM17 consists of ~750 amino acids and its domain structure has a pro-domain, a metalloprotease domain, a disintegrin domain, a cysteine-rich domain, and EGF-like domain, a transmembrane domain, and a cytoplasmic tail (Figure 4). The pro-domain has a chaperon function and inhibits catalytic activity. The pro-protein convertase furin cleaves the protein and consequentially the catalytic domain is de-repressed. ADAM17's activity can be regulated by several mechanisms including gene expression, intracytoplasmic and pericellular regulation, zymogen activation and inhibition by inhibitors. [32-35]

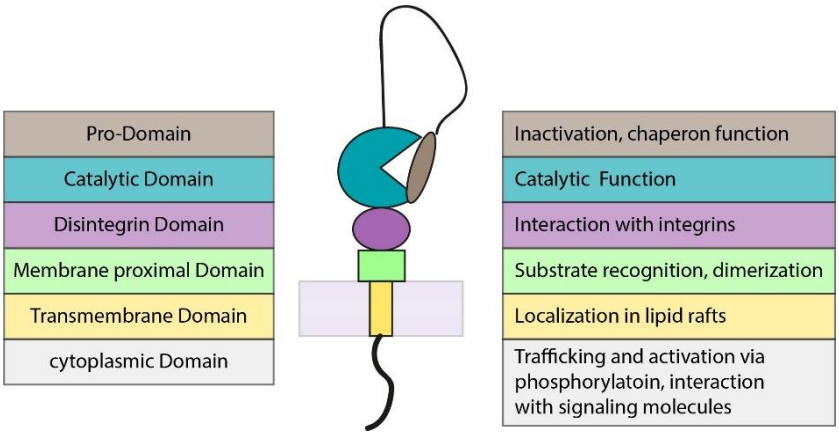

Figure 4. Structure and function of ADAM17
ADAM17 consists of six different domains, each having a distinct function. After removal of the pro-domain, the catalytic domain becomes activated. Figure adapted from [34]

1.4.2 ADAM17-mediated Signaling Pathways

The best characterized function of catalytically active metalloproteases is protein ectodomain shedding, which allows membrane-tethered factors to participate in auto- and/or paracrine signaling. A cleaved substrate can bind to its receptor, initiating downstream signaling. On the other hand, a receptor can be cleaved from the cell membrane and thus ligand-initiated signaling is stopped. Furthermore, ectodomain shedding is an important element of intercellular communication (Figure 5). [36]
ADAM17 is major convertase of ligands binding the Epidermal Growth Factor Receptor (EGFR)-related receptors such as TGF-α, Amphiregulin and Epiregulin. The EGF-receptor (ErbB1) is an important tyrosine kinase receptor whose downstream signaling regulates, among others, proliferation and migration and thus has crucial roles in development and cancer. [37] For example, the enhanced shedding of these ligands activates the ErbB receptors, also in distant cells. Aberrant ErbB receptor activity has been implicated in tumor development and progression.
Additionally, ADAM17 cleaves cell adhesion molecules that contribute to the cells

migratory and invasive capacity. For example, CD44, a binding molecule for hyaluronan, mediates migration and invasion of tumor cells and high expression and release of CD44 in the TME is correlated with an increased metastatic potential of tumor cells. Another adhesion molecule targeted by ADAM17, ALCAM (activated leucocyte adhesion molecule, CD166), is involved in several biological processes including hematopoiesis, immune response and migration and its expression and regulation may play a role in tumor progression. [29, 38]
Beyond the shedding of growth factors and its impact on neoplastic growth, ADAM17 is involved in the activation, recruitment and resolution of innate and adaptive immune responses. For example, Amphiregulin can induce proliferation and activation of regulatory T cells, thus also has an immune-suppressive function. [39]
The ubiquitous signaling pathways ADAM17 is involved in, with unique cell and tissue specific effects, underline the rational to investigate mechanisms for controlling ADAM17 activity.

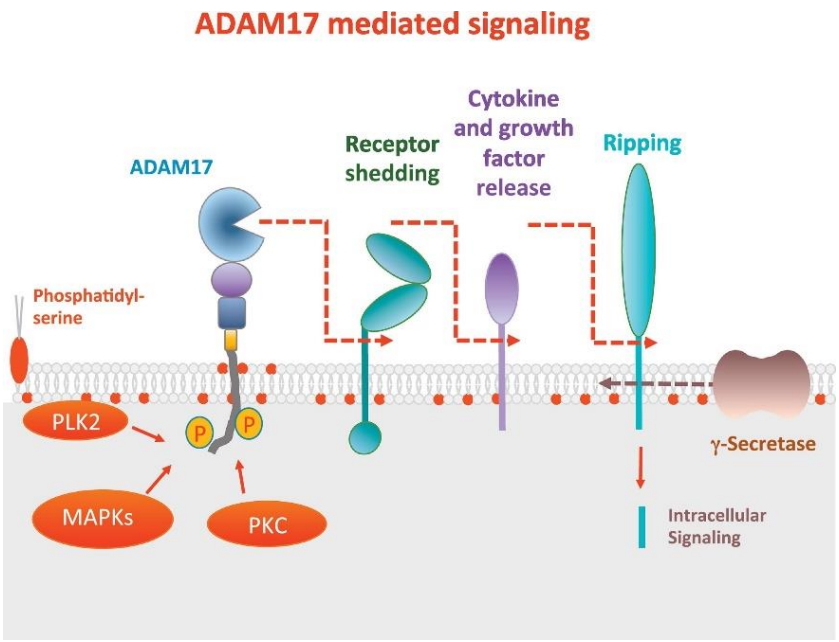

Figure 5. ADAM17-mediated signaling
Function of ADAM17 is regulated by phosphorylation of the cytoplasmatic tail by intercellular kinases. For ADAM17 activation, Phosphatidylserine is transferred to the outer leaflet of the membrane. ADAM17 processes over 80 single-spanning membrane proteins, including growth factors and cytokines that bind to receptors leading to activation of intracellular signaling pathways. [34]

1.4.3 ADAM17 in Cancer

ADAM17's involvement in pleiotropic events in tumorigenesis, including stimulation of proliferation and escape from immune surveillance (Figure 6), has led to studies identifying ADAM17's response to different treatment modalities. For example, ADAM17 activity is increased in cells treated with chemotherapeutic agents (fluorouracil), resulting in growth factor shedding, growth factor receptor activation and drug resistance. [40] Recently, it has been proven that in response to ionizing radiation, the furin-mediated activation of ADAM17 is promoted in NSCLC cells with increased shedding of ADAM17 substrates, contributing to an IR-induced stress response in these cells. [41]

ADAM17 overexpression has been linked to increased proliferation, invasiveness and poor prognosis in several different cancer types [42-44] which is why it has become an attractive therapeutic target. By inhibiting ADAM17 activity in tumors, proliferation and invasion can potentially be diminished and it may support immunosurveillance, all in all helping to control tumor progression and improving the treatment outcome.

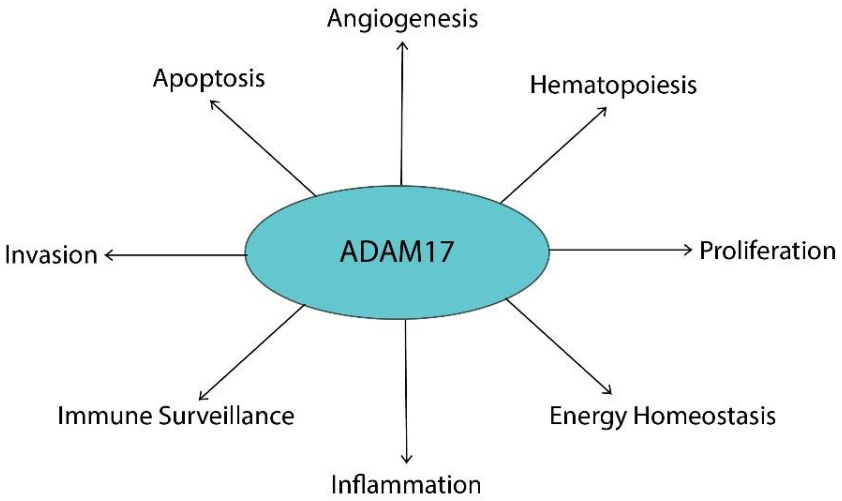

Figure 6. Multiple Processes are affected by ADAM17
ADAM17 displays a wide array of actions, systemic or cell-type specific. Focusing on cancer, ADAM17 influences eight processes which are actively involved in cancer progression.

1.5 AIM

Radiotherapy has been used to treat patients for almost a century. Its cytotoxic insult on the DNA is the main reason it has been used and extensively studied.[11, 45] Ionizing radiation also induces a multilayered stress response including auto- and paracrine factors that are released into the tumor microenvironment. These intra- and intercellular processes can co-determine treatment response and eventually treatment outcome. [18, 46]

The family of matrix metalloproteases are important orchestrators of shedding proteins on the outer side of the cell membrane. They are important players of auto- and paracrine signaling within the primary tumor, the tumor microenvironment, secondary distal tumors and metastases, and therefore represent a promising target in the field of radiobiology. To date, only limited comprehensive analyses have been performed with regard to the role of an irradiation-regulated serum proteome (secretome), in particular to a specific sheddase of interest. [47]

ADAM17, a disintegrin and metalloproteinase, drives pleiotropic pathways and increased ADAM17 expression is associated with aggressive progression of tumor growth and poor prognosis [31, 32, 39, 48]. Due to its broad spectrum of activity, ADAM17 represents an ideal target for combined treatment with IR. Previous results from our group identified ADAM17 as an important resistance mediator in response to IR. Using a potent ADAM17-inhibitor (TMI-005), radiosensitivity was increased. [41] These findings directed us to the goal to uncover the underlying molecular mechanisms of ADAM17 mediated radiation sensitization and its role in auto- and paracrine signaling and provide the baseline for my master thesis.

Precisely, my master thesis aims to investigate two different aspects:

a) Characterize the tumor cell's response to IR upon ADAM17-downregulation *in vitro*

To study the underlying mechanism of ADAM17-mediated resistance, we generate inducible ADAM17-knockdown cells using short-hairpin RNA (shRNA). The decision to choose an inducible shRNA knockdown system over a complete gene knockout by CRISPR/Cas9 was based on the interest in the regulation of ADAM17 levels to control the secretome in response to irradiation rather than a complete gene knockout of ADAM17.

We aim to validate and use the inducible shRNA system in order to identify biochemical and cell-biological mechanisms altered by the ADAM17 downregulation (e.g. protein level and activity). Furthermore, we will identify the efficacy of ADAM17-downregulation to sensitize cells towards ionizing radiation.

b) Quantitatively assess the ADAM17-dependent intercellular communication in response to IR

ADAM17 is a highly active metalloproteinase with many different target substrates. Many substrates are known to play a key role in tumor development and progression. We hypothesize that ADAM17 ligands are actively involved in intra- and intercellular communication responsible for tumor behavior, such as migration.

Using a transwell migration assay, we will evaluate the paracrine effect of a secretome generated by ADAM17-proficient cells as compared to a secretome produced by ADAM17-knockdown cells.

Together, these results will help us to uncover auto- and paracrine effects of ADAM17 and underline the rational for combining IR with a potent ADAM17 inhibitor to improve treatment outcome.

2 MATERIALS AND METHODS

2.1 METHODS

2.1.1 Cell Culture

The human non-small cell lung cancer (NSCLC) cell lines A549 and H358 were cultured in RPMI 1640 cell culture media supplemented with 10% (v/v) fetal calf serum, 1% (v/v) penicillin–streptomycin, and 1% (v/v) GlutaMAX (Thermo Fisher) at 37°C in 5% CO_2. All cell culture media and supplements were obtained from Gibco (Life Technologies).

Stable A549 and H358 cell lines established to express an ADAM17-targeting shRNA or a non-targeting control shRNA under the control of a doxycycline-inducible promoter were grown as indicated above. To induce shRNA expression, 500ng/ml Doxycycline (Dox) was added to the growth medium for at least 48h prior to initiation of the experiments.

To maintain a cell line in culture, 80-90% confluent dished were depleted of medium, washed with PBS and incubated in 0.25% EDTA-Trypsin to detach adherent cells. Cells were resuspended in an appropriate amount of cell culture medium and reseeded in a diluted variant.

2.1.2 Irradiation

Irradiation was performed using an Xstrahl 200 kV X-Ray unit at 100 cGy/minute. (Gulmay, Suwanee GA).

2.1.3 Proliferation Assay

Cells were incubated with Doxycycline for 72h and sham or 5Gy irradiated. The proliferative activity of tumor cells was assessed in 96- well plates with the colorimetric AlamarBlue assay (Biosource International). Through metabolization within the cell, AlamarBlue is irreversibly reduced to the pinkish and highly red fluorescent resorufin. The change in absorbance is measured and indicates the relative proliferative activity. Exactly 4h prior measuring the absorbance, 10µl AlamarBlue was added to the corresponding wells. The absorbance was measured at 590nm and 630nm by the Tecan GENios spectrophotometer.

2.1.4 Clonogenic Assay

Clonogenic cell survival was determined by the ability of single cells to form colonies in vitro as described [49]. Dox-induced cells were irradiated with different doses of IR and allowed to form colonies for 10 days. For fixation of colonies, cell media was removed, cells were washed with PBS and incubated for 20min with methanol/acetic acid 3:1. After removal of methanol/acetic acid, plates were left to dry overnight at RT. Next day, colonies were stained with crystal violet for 30min. Staining solution was removed, and plates were washed in water and left to dry. For evaluation, manual colony counting was assisted by a colony counting device (Gallenkamp). The plating efficiency (PE) was calculated by dividing the number of colonies by the number of

cells seeded on the non-irradiated control plate. Survival fraction (SF) was calculated as follow: $SF = \frac{colonies\ counted}{\frac{Cells\ Seeded}{PE}}$

2.1.5 TACE Activity Assay

ADAM17 activity was analyzed with the Innozyme™ TACE activity kit (Merck, CBA042). Procedure was performed as instructed by the company. In brief, Dox-induced cells were sham or 5Gy irradiated, and at different time points after irradiation, cells were harvested in Cytobuster™ Protein Extraction Reagent. After normalization of the protein concentration (250ng/ul), samples (and control) were loaded on the precoated 96-well plate and incubated for 1h at RT with gentle shaking. Following a wash step, TACE substrate was added and the sealed plate was incubated 4-5h at 37°C with gentle shaking. Fluorescence was measured at an excitation wavelength of ~324nm and an emission wavelength of ~405nm.

2.1.6 Western Blotting

Samples were prepared by scraping them off in sodium dodecyl sulfate (SDS) sample buffer and heating for 5min at 95°C (Thermomixer compact, Eppendorf). Protein concentration was measured (NanoDrop 1000 Spectrophotometer, Thermo Scientific), normalized to ~ 1mg/ml and stored at -20°C.

All samples were analyzed using SDS-polyacrylamide gel electrophoresis (PAGE). Usually, a running gel with a final concentration of 7.5% of acrylamide was prepared (see Table 1 for detailed description). 50 µg of samples were loaded into the corresponding wells and a current of 50mA was applied. The gel was the blotted onto a polyvinylidene fluoride (PDVF) membrane at 60V for 1h. The PVDF membrane was blocked with 5% blotting-grade blocker milk powder in TBS-Tween20 0.1% buffer for 30min at RT. The membrane was probed with the primary antibodies (see Table 5) over night at 4°C with rotation at 200 RPM. Next day, the membrane was washed 3x for 10min with TBS-T buffer. The HRP-conjugated secondary antibody was diluted in 5% milk (see Table 2) and incubated shaking for 1h at RT. The membrane was washed 3x with TBS-T before the antibody was detected by chemiluminescence with the Vilber Lourmat Fusion FX Detector.

Table 1. Tris-Glycine SDS-Polyacrylamide Gel Composition

	Running Gel (7.5%)	**Stacking Gel**
ddH_20 (ml)	7.5	3.01
1.5M Tris pH 8.8 (ml)	3.75	-
0.5M Tris pH 6.8 (ml)	-	1.25
30% acrylamide (37.5:1) (ml)	3.75	0.65
10% APS (µl)	50	25
TEMED (µl)	10	5

Table 2. Primary and Secondary Antibodies used for Western Blotting

Antibody	Supplier	Species	Dilution
Primary Antibodies			
Anti-β-actin	Sigma Aldrich	Mouse	1:1000
Anti-ADAM17	Cell Signaling	Rabbit	1:1000
Secondary Antibodies			
Anti-Mouse	GE Healthcare	Sheep	1:5000
Anti-Rabbit	Santa Cruz	Mouse	1:7500

2.1.7 Enzyme-Linked Immunosorbent Assay (ELISA)

Cells were seeded in 6-well plate and allowed to attach for at least 12h. Medium was replaced with fresh RPMI (full) prior to irradiation. Plates were incubated at 37°C, 5% CO_2, for 24h. Thereafter, medium was harvested and filtered through a 0.45 µm filter and cells were counted with a Neubauer Chamber. To measure Amphiregulin concentration in media, manufacturer's protocol was followed (Duoset® Human Amphiregulin, DY262, R&D Systems). In brief, capture antibody was diluted to working concentration in PBS, added to 96-well plate and incubated overnight at 37°C, 5% CO_2. Next day, plate was washed and blocked for 1h with Reagent Diluent at RT. Following a wash step, standards and sampled were added in triplicates and the sealed plate incubated for 2h at RT. Following another wash step, detection antibody was added, and the sealed plate was incubated for 2h at RT. Following a wash step, the streptavidin horse-radish peroxidase (HRP) and incubated for 20min at RT in the dark. Substrate A and B (1:1) were added and plate was incubated for 20min at RT in the dark. Stop solution was added and the absorbance was measured at 450nm with a wavelength correction at 540nm (Ultra Microplate Reader EL 808, Bio-Tek Instruments, Inc., Switzerland)

2.1.8 Flow Cytometry

A population of cells was prepared for flow cytometry by trypsinizing and filtering using standard protocol. Data were analyzed with FlowJo software (Ashland, OR). Cellular debris and dead cells were excluded by their light-scattering characteristics. Transduced A549 cells were gated according to intrinsic GFP or RFP expression as measured by a Becton Dickinson FACS LSRFortessa.

2.1.9 Transwell Assay

Transwell inserts (6.5 mm, 8um pores, Costar) were used to analyze the paracrine effect of ADAM17-secretome on A549 cell migration. Briefly, $1x10^5$ A549 cells (control/knockdown) were plated into the lower chamber of the transwell containing 1000µl complete RPMI medium and allowed to attach for a minimum of 12h. Thereafter, the medium was replaced with 1000µl RPMI supplemented with 1% (v/v) FBS, 1% P/S and 1% GlutaMax and the plate was sham or 5Gy irradiated and cultured for additional 6h. Next, $3x10^4$ A549 cells in 100µl RMPI medium (1% FBS) were seeded into the upper chamber of the transwell inserts. The coculture was maintained at 37°C in 5% CO_2 for 24h. For quantification, cells from the upper side of the insert were

scraped away with a cotton swap and inserts were then fixed in Methanol/Acetic Acid (75%/25%, v/v), dried and then stained with DAPI. Fluorescent microscopy pictures were taken (Axiovert 40 CFL, Zeiss with AxioCam MRc) and the migrated cells were counted manually.
In this set-up, either cell type (upper well/lower well) can be treated independently from the other, but the two cell types will be able to communicate via the shared cell medium (Figure 9). Migration of cells through the PET membrane (8µm) can be stimulated by various factors that are produced by cells residing in the lower compartment. For the current study, either cell type's secretome (control/knockdown) could be placed in the lower compartment and all cells (migrating/secretome producing) could be differentially irradiated (Figure 9 A-D).

2.1.10 Production of stable cell lines

2.1.10.1 Lentiviral Plasmid, shRNA Sequences and Vector Map

The plasmid vector *pRSITPRP-U6Tet-sh-PGK-TetRep-2A-TagRFP-2A-Puro* (Figure 7) was used to produce lentivirus for transduction of cell lines with inducible short-hairpin-RNA mediated downregulation of ADAM17. The vector contains the PGK promoter driving expression of a Tet repressor (TetR), red fluorescent protein (RFP) and puromycin resistance and the inducible short hairpin RNA (shRNA, (Table 3)) is driven by the U6-Tet promoter sequences. This Tet-On system initiates the transcription and processing of the shRNA by addition of doxycycline and thus an expressional downregulation of the target protein (Figure 8).[50, 51]

Table 3. shRNA nucleotide sequences

Name	Sequence
shADAM17_NonTarget	CAACAAGATGAAGAGCACCAA
shADAM17.2	GATCATCGCTTCTACAGATAC
shADAM17.3	CCTGGTTACAACTCATGAATT

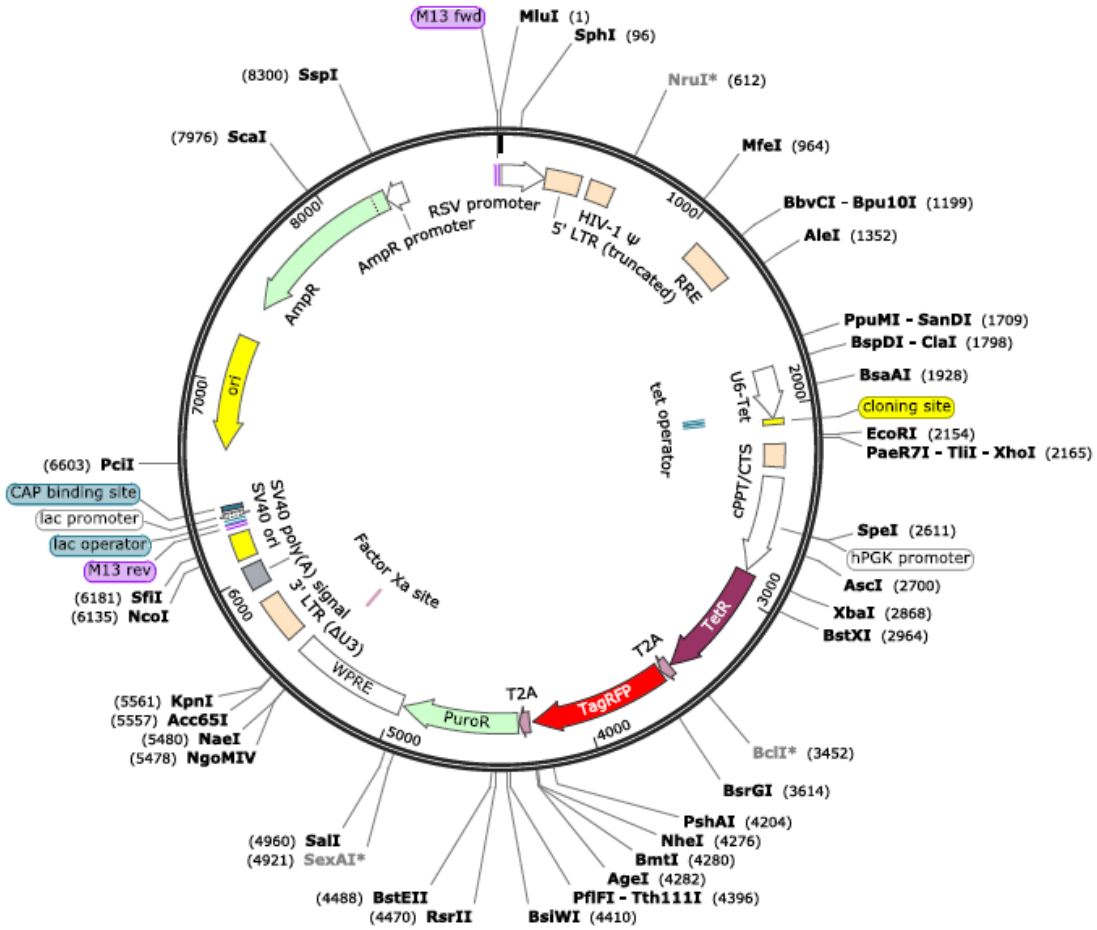

Figure 7. Vector-Map: pRSITPRP-U6Tet-sh-PGK-TetRep-2A-TagRFP-2A-Puro (8887bp)

2.1.10.2 Transformation of JM109 Competent E. coli Cells

The JM109 competent E. coli cells (Single-Use JM109 Competent Cells, >108cfu/µg, Promega) were thawed on ice. To 90 µL of cells, 50 ng of plasmid in a volume of 10 µL was added giving a total volume of 100 µL. The cells were incubated on ice for 30 min, 45 sec at 42°C (Heat Shock) and then 2 min on ice again. 900 µL of prewarmed lysogeny broth (LB) media was added and incubated shaking at 37°C for 1 h. Thereafter, the bacteria were streaked on LB-agar selection plates (containing the antibiotic ampicillin, 100ng/mL). The plates were incubated over night at 37°C. The next day, one colony of transformed E. coli cells was picked and incubated in 3 mL LB media with ampicillin (100ng/mL) overnight at 37°C with shaking. The 3 mL cultures were transferred to 250 mL LB media with ampicillin (100ng/ml) and incubated with shaking at 37°C overnight.

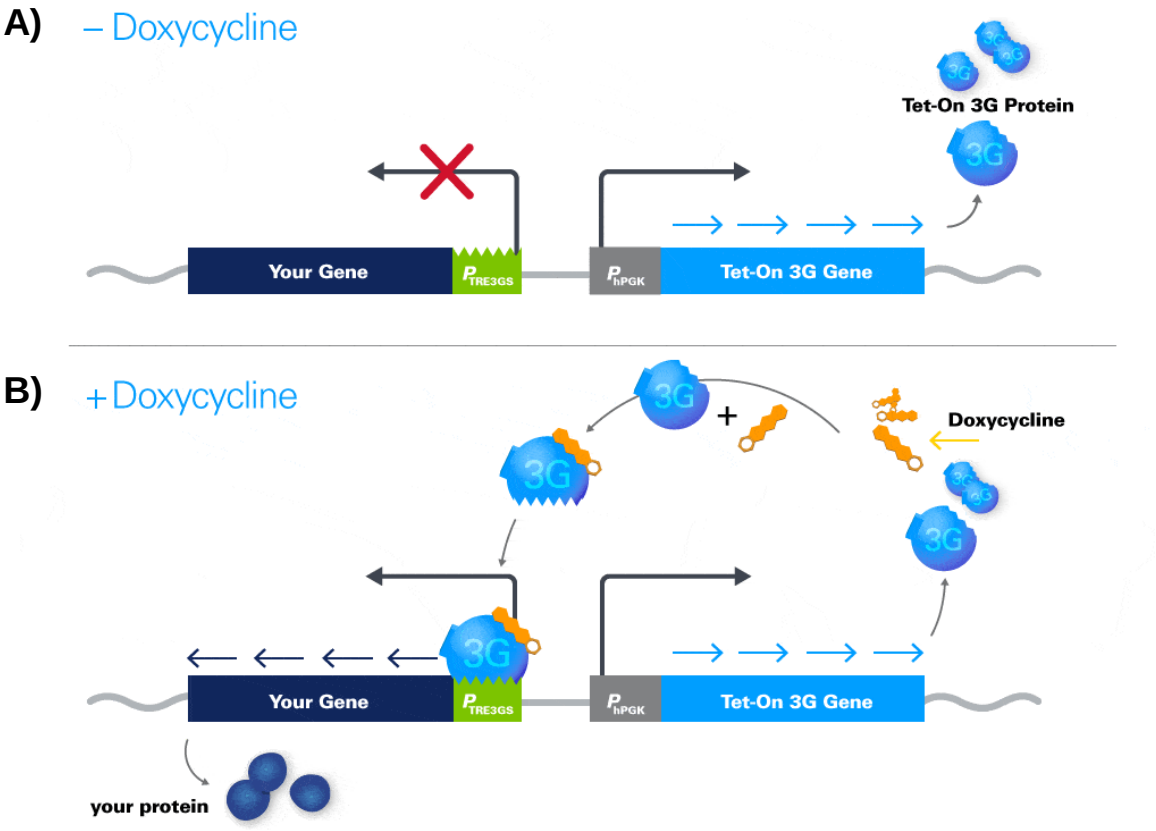

Figure 8. Tet-On System
The Tet-On system allows the regulation of a gene of interest (G.O.I) by administration of tetracycline (or its derivatives like Doxycycline). This quantitative and temporal control of gene expression by an exogenous effector molecule reduces adverse effects and improves the safety of gene therapy.
The Tet-On system is based on the reverse Tet-Repressor protein (Tet-On 3G Protein) and tet operator (P_{TRE3G}) DNA elements. The Tet-On 3G Protein does not bind the P_{TRE3G} in the absence of the effector (Doxycycline) (**Fig. A**). Binding of Doxycycline triggers a conformational switch in Tet-On 3G Protein which allows P_{TRE3G} binding (**Fig. B**). The subsequent activation of the promoter drives expression of the downstream positioned gene (G.O.I).
An ideal Tet-On system has low background activity in the absence and high activity in the presence of doxycycline. (Figure adapted from [50])

2.1.10.3 Plasmid Amplification and Purification

Plasmid purification was performed following the manufacturer's protocol and using the HiSpeed® Plasmid Midi Kit (25) (Qiagen, Ref. 12643).

2.1.10.4 Transfection of HEK293T Cells and Production of Lentiviral Particles

HEK293T cells (human embryonic kidney 293 cells expressing the large tumor (T-) antigen from the SV40 virus) were transfected with the plasmid to generate lentiviral particle for later transfection of NSCLC cell lines. The manufacturer's protocol (Cellecta: Packaging, Titering and Transduction of Lentiviral Constructs, 2015) was

followed. In brief, the day before the transfection, 4 x 10^6 HEK cells were plated in a 75 cm^2 flasks and incubated over night at 37°C to have them at ~70% confluency the next day. For each lentiviral construct, 20 µl (to a final concentration of 10 µg/mL) of the ready-to-use packaging plasmid mix was mixed with 4 µL (to a final concentration of 2 µg/mL) of the plasmid. 1 mL of Opti-MEM media was added and incubated at RT for 15 min. 30 µl lipofectamine was mixed with 1 mL Opti-MEM media. 1 ml of the lipofectamine mix was added to the plasmid mixture and incubated at RT for 15 min. 2 mL of the plasmid/lipofectamine mix was added to each 75 cm^2 flask. The flasks were incubated at 37°C for overnight. The media was changed to fresh DMEM the next morning and cells were incubated at 37°C until the next day evening. The virus containing media was collected, spun down and the supernatant was filtered through a 0.45 µm PES low protein binding filter.

2.1.10.5 Transduction of New Target Cells

Target cells were seeded at 2.4 x 10^6 in a 75cm^2 flask. When cells were around 80% confluent, 7ml of RPMI was mixed with 7ml of viral supernatant and 1.5 µl of polybrene (5µg/ml) and added to target cells. Flasks were incubated over night at 37°C. Next day the medium was removed and replaced by fresh RPMI medium and incubated 24h. Then, the medium was changed to RPMI containing 1ug/ml puromycin, to select for cells that successfully integrated the transgene. Cells were selected over a week (medium change every second day) and successful transduction was controlled by checking the GFP/RFP signal under a fluorescence microscope (Axiovert 40 CFL, ZEISS with AxioCam MRc).

2.1.11 Statistical Analysis

Statistical analysis was performed with GraphPad Prism 7.04. Data is represented as mean ± standard deviation of the mean (SEM). Significance was measured by unpaired student t test. $P \leq 0.05$ was considered significant.

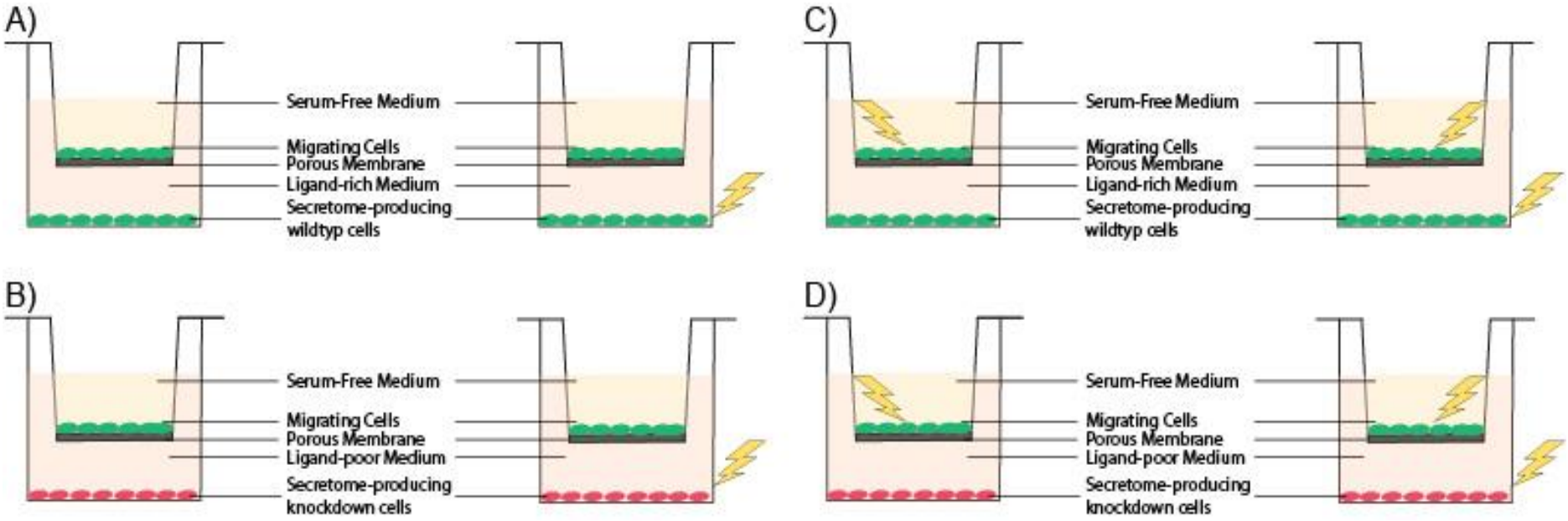

Figure 9. Schematic representation of the transwell migration experiment

(**A**)-(**D**) Either cell type (upper well/lower well) can be treated independently from the other, but the two cell types will be able to communicate via the shared cell medium. Migration of cells through the PET membrane (8µm) can be stimulated by various factors that are produced by cells residing in the lower compartment. For the current study, secretome producing cells (control/knockdown) could be placed in the lower compartment and all cells (migrating/secretome producing) could be differentially irradiated

2.2 Cell Lines

Table 4. Cell lines [52]

Cell line	Tissue	Mutation		
		EGFR	*KRAS*	*TP53 status*
A549	Human NSCLC	wt	mut	wt
H358	Human NSCLC	wt	mut	mut
HEK 293 T	Human embryonic kidney cells (transformed with large T antigen)			

2.3 Buffers and Solutions

Table 5. Buffers and Solutions

10 % APS	**100mg/ml APS in ddH_20**
Acrylamide solution 30 %	29.2g/l Acrylamide, 0.8g/l Bis-Acrylamide in ddH_20
Running Gel buffer	1.5M Tris, 0.1% SDS in ddH_20, pH 8.8
SDS running Buffer	0.3% Tris, 1.44% Glycine, 0.15% SDS
SDS-sample buffer	125mM Tris, 4% SDS, 20% Glycerol in ddH_20, pH 6.8
Stacking Gel buffer	0.5M Tris, 0.1% SDS in ddH_20, pH 6.8
TBS-T	0.1M Tris, 150nM NaCl, 0.1% Tween-20 in ddH_20, pH 8.0
Transfer Buffer	100nM Tris, 192nM Glycine, 10% methanol

2.4 Chemicals

Table 6. Chemicals

0.5 % Trypsin-EDTA 10x	**Gibco by Life Technologies**
2-mercaptoethanol	Sigma - Aldrich
Acetic Acid (glacial) 100 %	Emsure®, Merck
Acrylamide	Sigma
Agarose	Sigma
Ampicillin Sodium Salt	Sigma
APS Ammonium Persulfate	BioRad
Bis-Acrylamide	BioRad
Blotting-Grade Blocker (Milk Powder)	BioRad
Boric Acid	Fluka
BSA (Bovine Serum Albumin)	Sigma
Coomassie Brilliant Blue	Sigma

Crystal Violet	Merck
Custom Lentiviral shRNA Constructs (Plasmids)	Cellecta
Cytobuster™ Protein Extraction Reagent	Millipore, Novagen®, Merck
DAPI	Sigma - Aldrich
Dimethyl sulfoxide (DMSO)	Sigma - Aldrich
DMEM Media	Gibco by Life Technologies
Doxycycline (Dox)	Sigma
ECL™ Anti-Mouse IgG Horseradish Peroxidase linked whole Antibody (from sheep)	GE Healthcare
ECL™ Anti-Rabbit IgG Horseradish Peroxidase linked F(ab)$_2$ fragment (from donkey)	GE Healthcare
ECL™ Western Blotting Detection Agents	Amersham™, GE Healthcare
Ethanol (EtOH)	Merck
FCS (Fetal calf serum)	Gibco by Life Technologies
Gelred (1000x)	Biotium
Glycerol	Sigma
LB Agar Powder, Lennox L Agar	Invitrogen
L-glutamine 200 mM (100x)	Gibco by Life Technologies
Lipofectamine 2000	Invitrogen
Methanol (MeOH)	Morphisto
Monoclonal mouse anti-β-Actin	Sigma Aldrich (#A5441)
Opti-MEM	Gibco by Life Technologies
PBS pH 7.2	Kantonsapotheke Zürich
Pen Strep (Penicillin/Streptomycin)	Gibco by Life Technologies
Polybrene	Sigma Aldrich
Polyclonal rabbit anti-ADAM17	Calbiochem (#PC491)
Polyclonal rabbit anti-Akt	Cell Signaling Technology (#9272)
Potassium Chloride (KCl)	Fluka Biochemika
Puromycin Dihydrochloride	Sigma
Ready-to-use-packaging Plasmid Mix	Cellecta
RPMI 1640 Media	Gibco by Life Technologies

sodium dodecyl sulfate (SDS)	Sigma
Sodium Chloride (NaCl)	Sigma-Aldrich
Tetramethylethylenamidin (TEMED)	BioRad
Trizma® base	Sigma
Tween® 20	Sigma

3 RESULTS

3.1 LENTIVIRAL PLASMID AMPLIFICATION AND TRANSDUCTION OF NEW CELL LINES

The addition of drugs inhibiting ADAM17 activity strongly enhanced the efficacy of IR in *in vitro* and *in vivo* studies as published by Sharma et al. [41]. To study the underlying mechanisms of ADAM17-mediated radioresistance, a lentiviral plasmid system was used to generate new doxycycline-inducible ADAM17-knockdown NSCLC cell lines.
In this project, the two NSCLC cell lines A549 and H358 were transduced with the lentiviral particles. For each cell line, three different constructs, containing a different shRNA, were used: a non-targeting short hairpin (referred to as shNT or control), and two different short hairpins targeting ADAM17 (referred to as shADAM17.2/shA17.2 and shADAM17.3/shA17.3). (see Table 3 for sequences)
Cells were selected with puromycin containing medium and subsequently, A549 cells were analyzed by flow cytometry to verify the expression of GFP (shNT) or RFP (shADAM17). Flow cytometry scatter blots show that approximately 90% of A549 cells containing the non-target shRNA express the green fluorescent protein (Figure 10A) and approximately 90% of A549 cells containing the ADAM17-targeting shRNA (shADAM17.2 resp. shADAM17.3) express the red fluorescent protein (Figure 10 B+C)

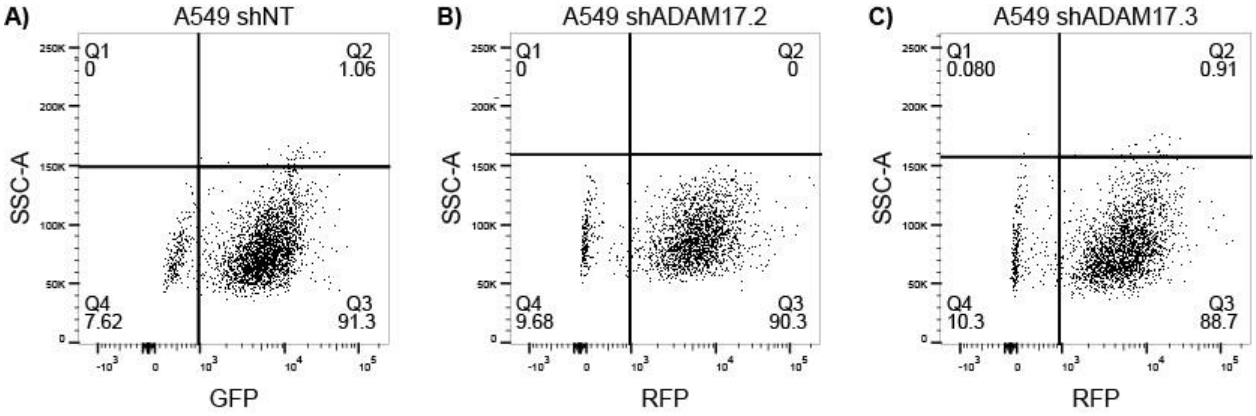

Figure 10. Transduced A549 cells stably express the inserted vector as determined by GFP/RFP expression
(**A**) Flow Cytometry scatter plot analysis showing GFP-expressing A549 cells containing the shNT. (**B**)+(**C**) Flow Cytometry scatter plot analysis showing RFP-expressing A549 cells containing the ADAM17 targeting shRNAs (shADAM17.2 (B) resp. shADAM17.3 (C)).

3.2 THE INDUCTION OF ADAM17-DIRECTED SHRNA REDUCES ADAM17 PROTEIN LEVEL AND ACTIVITY

After the successful transduction and selection of the new stable cell lines, the efficiency of the Tet-On System to downregulate ADAM17 was tested and analyzed by Western blot.

In doxycycline-untreated cells (-), no differences in ADAM17 protein level were observed between the control cells and the cells harboring the shADAM17 constructs in cell lines A549 and H358. After adding doxycycline to the cells (+), protein levels of ADAM17 were decreased in cells transduced with shADAM17.2 and shADAM17.3 constructs. While the two constructs showed similar knockdown efficiency in H358 (Figure 11B), the shADAM17.2 was more efficient compared to shADAM17.3 in A549 cells (Figure 11A). Protein levels in doxycycline-treated cells containing the non-targeting shRNA were unaffected. (Figure 11 A+B)

The activity of ADAM17 protease in doxycycline-treated A549 and H358 cells was assessed with a protease activity assay. In line with protein downregulation, protease activity was reduced in A549 and H358 cells harboring the shADAM17 constructs relative to control cells. The activity reduced to approximately 30% in A549 (both shADAM17 constructs, Figure 11C) and to approximately 40% (shADAM17.2, Figure 11D, light red) or 80% (shADAM17.3, Figure 11D, dark red) in H358 cells relative to corresponding control cells. The activity reduction is still apparent following irradiation (5Gy), being at approx. 50% in A549 cells (both constructs, Figure 11E) and at 10% (shADAM17.2, Figure 11F, light red) resp. 50% (shADAM17.3, Figure 11F, dark red) in H358 cells relative to the corresponding control cells.

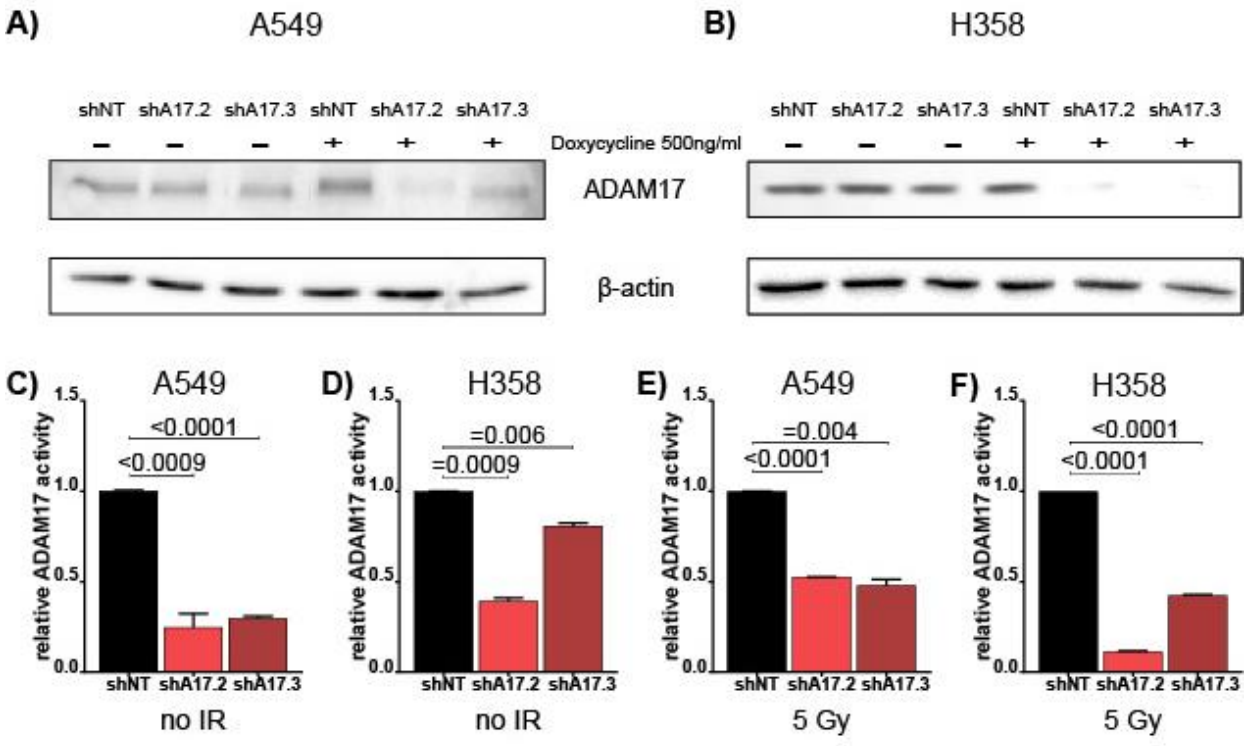

Figure 11: Tet-On System results in decreased ADAM17 protein levels and activity
(**A**) + (**B**) A549 or H358 cells were not induced (-) or induced (+) with Dox (500ng/ml) 72h prior to lysis. Whole cell lysates were loaded onto a 7.5% gel and subjected to analysis by immunoblotting using the indicated antibodies. (**C**) + (**D**) A549 or H358 cells were induced with Dox (500ng/ml) 72h prior to sham or 5Gy irradiation. 24h thereafter, relative ADAM17 enzyme activity was determined. Data displayed as relative mean ± SEM and analyzed with unpaired student *t* test. Significance level $p<0.05$.

3.3 ADAM17 ENZYME ACTIVITY INCREASES UPON IR

Posttranslational modifications, and not increased protein expression, result in higher ADAM17 enzyme activity following irradiation.[41] In A549 control cells, the ADAM17 protease activity increased in a time dependent manner, reaching its maximum with an approximately 2.5-fold increase 24h after irradiation (Figure 12A). Neither in the cells harboring the shADAM17.2 construct (Figure 12B) nor in cells with the shADAM17.3 construct (Figure 12C), a time dependent increase in protein activity could be observed. In H358 control cells, the ADAM17 protease activity increased concomitantly over time after irradiation, reaching its maximum with an approximately 1.5-fold increase 6h after irradiation. After 6h, the activity decreased to basal level (Figure 12D). In contrast, the cells harboring the shRNA constructs targeting ADAM17 (shADAM17.2 resp. shADAM17.3) did not show an increase in ADAM17 protease activity following irradiation (Figure 12E+F).

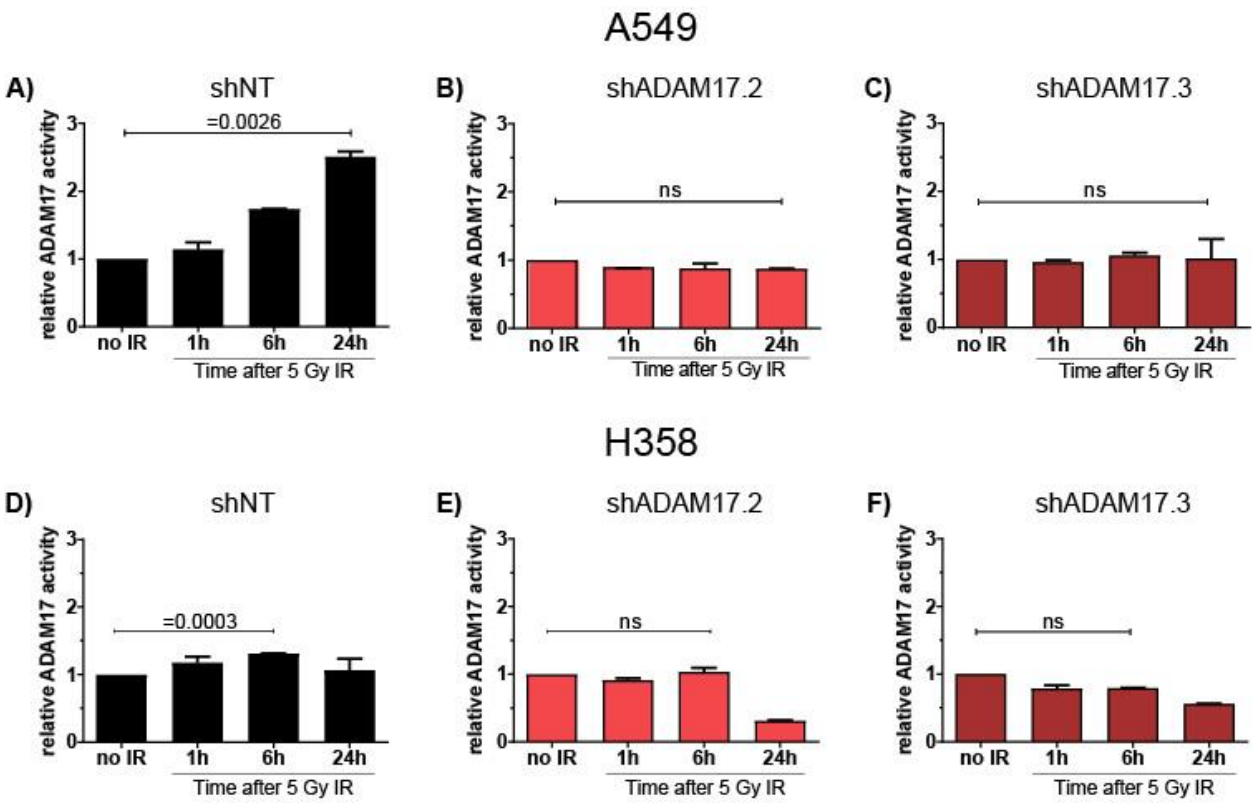

Figure 12. ADAM17 activity increases over time after irradiation

(**A**) - (**F**) Dox-induced A549 or H358 cells were irradiated with 5Gy. ADAM17 activity was determined before and at different timepoints after irradiation. Data displayed as relative mean ± SEM and analyzed with student *t* test. Significance level $p<0.05$.

3.4 ADAM17 DOWNREGULATION RESULTS IN DECREASED LIGAND SHEDDING

The semi-quantitative large-scale secretome analysis performed by Sharma et al. showed increased secretion of several substrates of ADAM17, in particular Amphiregulin and ALCAM. [41]

The amounts of Amphiregulin in culture media of non-irradiated or irradiated A549 shNT resp. shADAM17 cells were measured by an Amphiregulin-directed ELISA.

There was significantly less secreted Amphiregulin in the supernatant of ADAM17-knockdown cells compared to control cells (Figure 13A).
However, when the Amphiregulin levels in supernatant of non-irradiated cells were compared to supernatant of irradiated cells, a significant increase was seen not only in the shNT control cells, but also in the shADAM17 cells, albeit it started from a lower baseline level (Figure 13B).

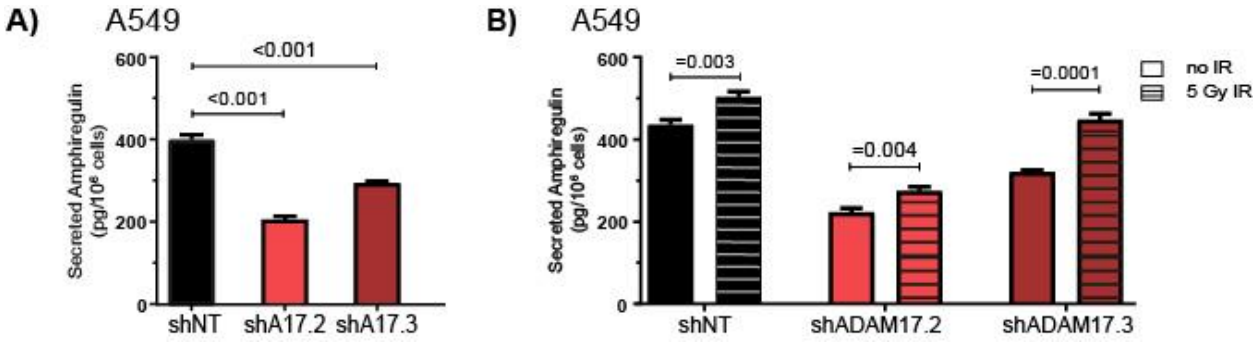

Figure 13. The secretion of the ADAM17 substrate Amphiregulin is enhanced following IR

(**A**)+(**B**) A549 cells were sham or 5Gy irradiated and 24h thereafter the supernatant was collected. The amount Amphiregulin in the supernatant of A549 control resp. knockdown cells was measured by Enzyme-Linked Immunosorbent Assay (ELISA). Data displayed as mean ± SEM and analyzed with unpaired *t* test. Significance level $p<0.05$.

3.5 ADAM17 DEPLETION SENSITIZES CELLS TOWARDS IONIZING RADIATION

The previous results demonstrated that downregulation of ADAM17 by shRNA decreases the protein level, the total protein activity and the level of secreted ligands. As a functional readout and to study whether reduced ADAM17 protein activity sensitizes cells to IR, proliferation and clonogenic assays were performed.
A549 shNT cells displayed a higher proliferative activity compared to cells containing the shADAM17.2 but not to cells containing the shADAM17.3. The difference in proliferative activity was amplified in a dose-dependent way if cells were subjected to irradiation (Figure 14A). Depletion of ADAM17 in H358 cells had no effect on the basal proliferative activity, as there was no difference detectable compared to control H358 cells. Following irradiation with 3Gy or 6Gy, ADAM17-depletion resulted in decreased proliferative activity also in H358 shADAM17 cells compared to control cells (Figure 14B).

In the clonogenic assay performed with the A549 control/knockdown cells, the survival fractions are represented as a function of dose. A549 shADAM17 cells had lower clonogenic survival compared to control cells when treated with increasing doses of ionizing radiation above 4Gy (Figure 14C).

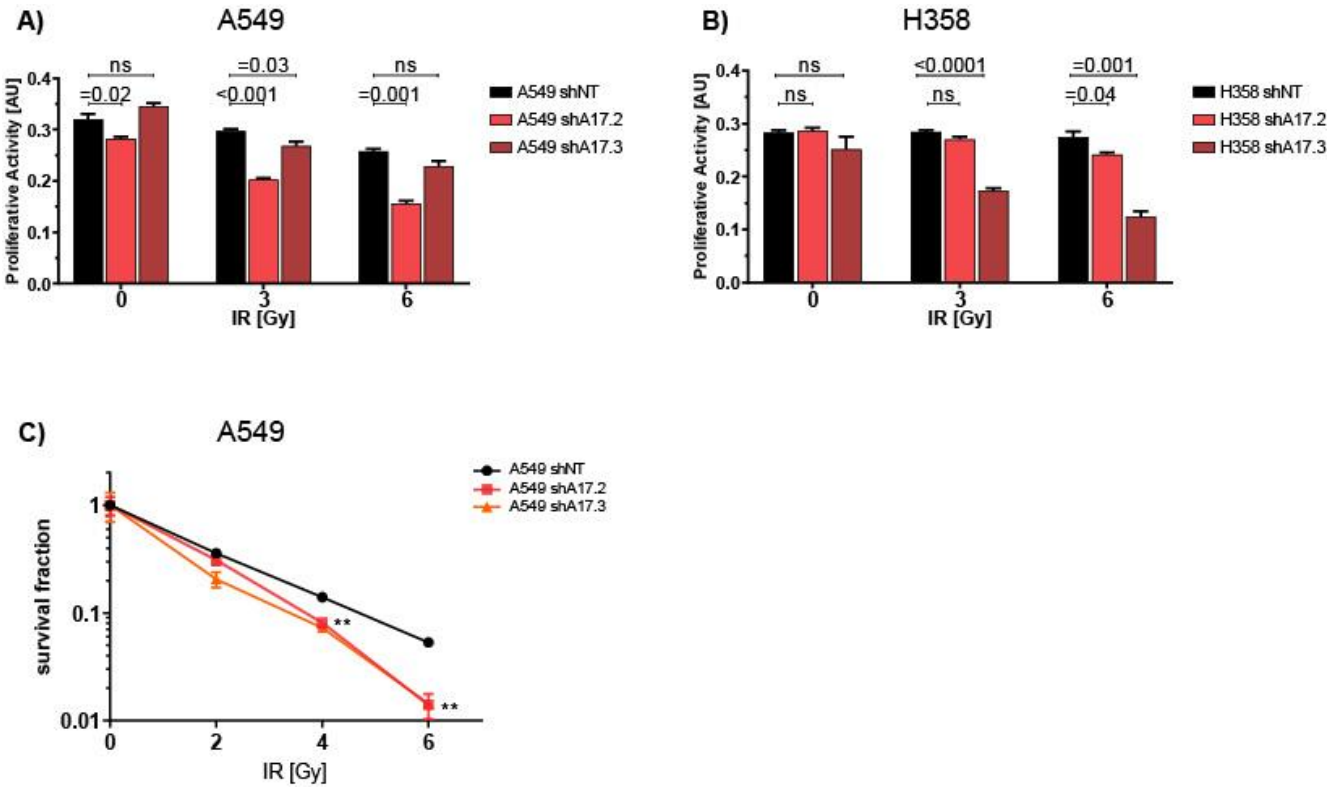

Figure 14. ADAM17 depletion sensitized cells towards IR resulting in lower proliferative activity and decreased clonogenicity

(**A**) + (**B**) Doxycycline induced cells (A549 and H358) were 0 Gy, 3 Gy or 6 Gy irradiated and 72h later their proliferative activity was measured using the AlamarBlue Cell Viability Reagent. Data displayed as mean ± SEM and analyzed with unpaired t test. (**C**) A549 cells were irradiated at increasing doses (0, 2, 4, 6 Gray) and following their capacity to form colonies was analyzed. Data displayed as mean ± SEM and analyzed with student t test. *p<0.05, **p<0.005, ***p<0.001

3.6 ADAM17-CLEAVED FACTORS ARE INVOLVED IN INTERCELLULAR COMMUNICATION

The previous results demonstrated that ADAM17s enzyme activity is upregulated in response to IR, leading to more secreted substrates. In addition, cells with normal ADAM17 protein level are more resistant to ionizing radiation compared to cells with decreased ADAM17 protein levels.

Multiple studies have already assessed the effect of ADAM17 on migration and concluded that ADAM17 knockdown cells show decreased migration and invasion capabilities.[30, 53] To test if ADAM17 is involved in intra- and intercellular communication and influences the cell behavior by autocrine and paracrine signaling, respectively, a transwell migration assay was performed. In line with former studies, the A549 cells harboring the shADAM17 construct used in this study showed decreased migration capabilities towards complete medium compared to control cells. (Figure 15A-C)

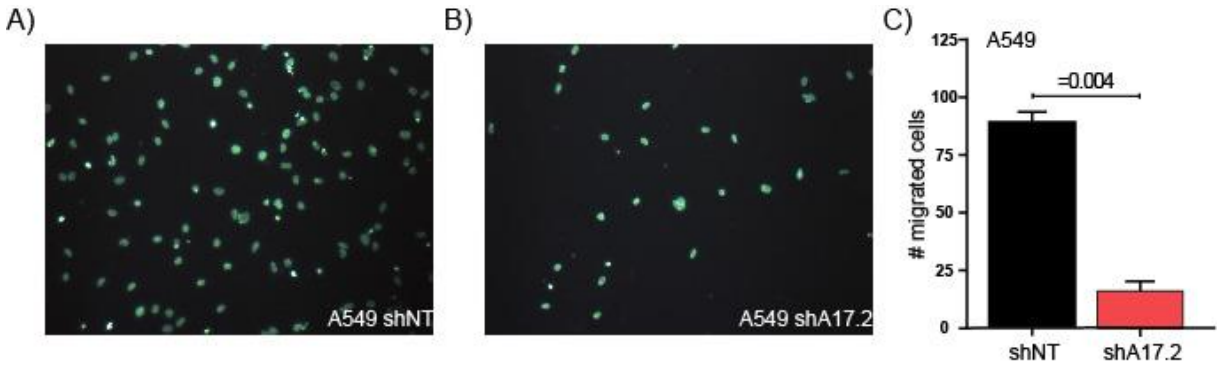

Figure 15. ADAM17 downregulation reduces migration capacity of A549 cells
(**A**) + (**B**) A549 shNT or shADAM17.2 could migrate towards complete medium. After 24h, cells were fixed, stained with DAPI and analyzed under the microscope. Representative microscopy pictures show migration of A549 shNT cells (A) and A549 shA17.2 cells (B). (**C**) For each condition, two separate membranes (n=2) were analyzed and combined data is represented in the bar graph. Data displayed as mean ± SEM and analyzed with student *t* test. Significance level $p<0.05$

Next, the migration capacity of A549 shNT cells towards an ADAM17-dependent secretome was studied.

A549 shNT cells could migrate towards a secretome produced either by A549 shNT or shADAM17 cells (Figure 9A+B, left) Interestingly, A549 shNT cells migrated better towards a secretome produced by control cells than by A549 shADAM17 (150.5±30.5 cells/field versus 94±20.8 resp. 62± 22 cells/field, Figure 16A-C, G).
Additionally, it was evaluated whether the increase of shed ligands in the secretome upon irradiation of A549 shNT or shADAM17 cells (as demonstrated in Figure 13) can amplify the difference between the migration towards secretome of shNT or shADAM17 cells respectively. For this, shNT cells could migrate towards a secretome coming from irradiated (5Gy) A549 shNT or shADAM17 cells (Figure 9A+B, right). Similar to the non-irradiated secretome, the A549 shNT cells migrated likewise less towards the secretome of A549 shADAM17 cells compared to secretome of A549 shNT cells (148.5±85.2 versus 132±12 resp. 72±16.4), although no further increase in the differential migration was observed ($p>0.05$). (Figure 16D-F, H).

The migration behavior of non-irradiated cells differs from irradiated cells. [9, 54] Therefore, the differential migration of irradiated cells was assessed, to see if the same pattern of migration as in non-irradiated cells can be observed. Irradiated (5Gy) A549 shNT cells could migrate towards a secretome coming from either non-irradiated or irradiated shNT or shADAM17 A549 cells (Figure 9C+D).
Irradiated shNT cells migrated less towards the secretome of ADAM17-knockdown cells compared to secretome of shNT cells (117.2±12.4 versus 90.5±11.8 resp. 57.5±25.2, Figure 17A-C, G) and irradiation of the secretome producing cells did not change the differential migration pattern (163±34 versus 72.5±31.2 resp. 80±28.6, Figure 17D-F, H). Of note is however the almost 1.4-fold increase in migration (117±12.4 versus 163±34, $p=0.01$) towards a secretome produced by irradiated shNT cells as compared to secretome of non-irradiated shNT cells (Figure 17A+D, G+H black bar).

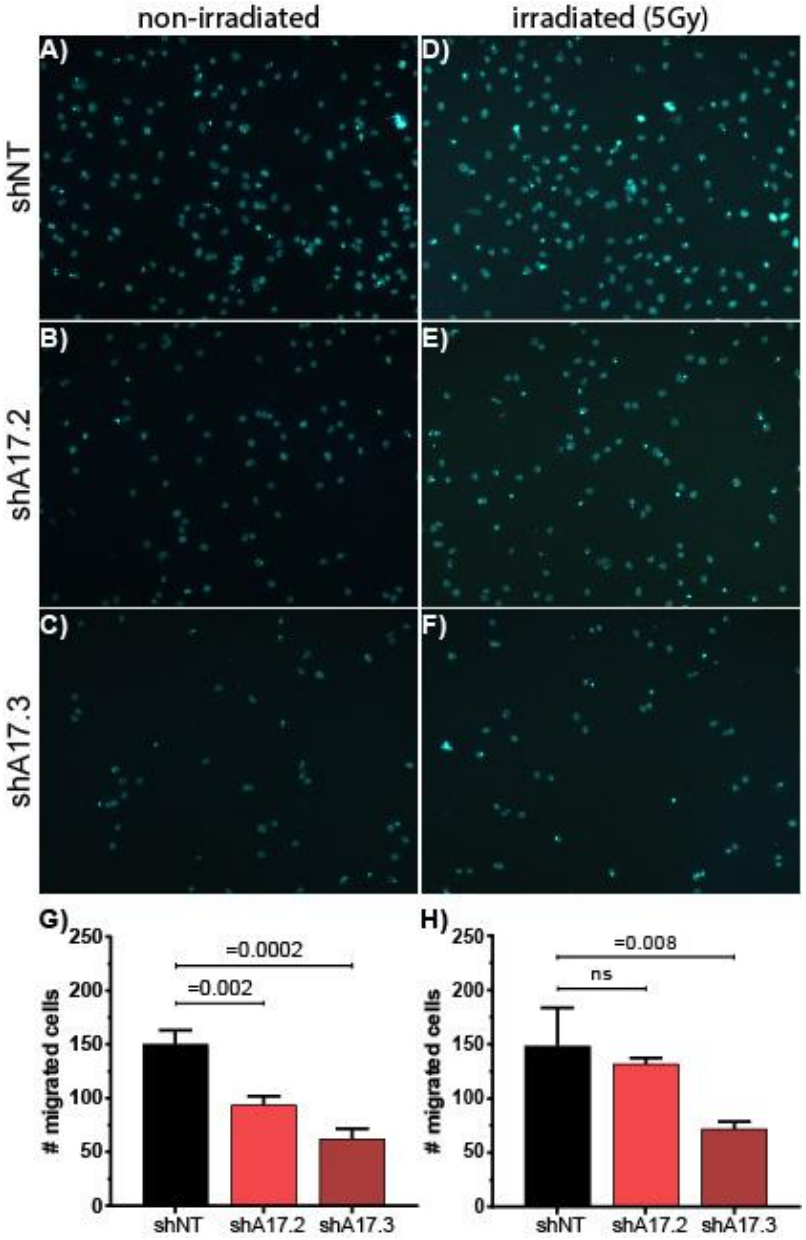

Figure 16. A549 shNT cells migrate significantly less towards the secretome of ADAM17-knockdown cells
(**A**)-(**C**) shNT A549 cells could migrate towards secretome of non-irradiated control (A) resp. ADAM17-knockdown cells (B+C). (**D**)-(**F**) shNT A549 cells could migrate towards secretome of irradiated control (D) resp. ADAM17-knockdown cells (E+F). (**G**)+(**H**) For each condition, two separate membranes (n=2) were analyzed and combined data is represented in the bar graph. (G) Data obtained from migration towards non-irradiated secretome (A-C). (H) Data obtained from migration towards irradiated secretome (D-E). Data displayed as mean ± SEM and analyzed with student *t* test. Significance level $p<0.05$

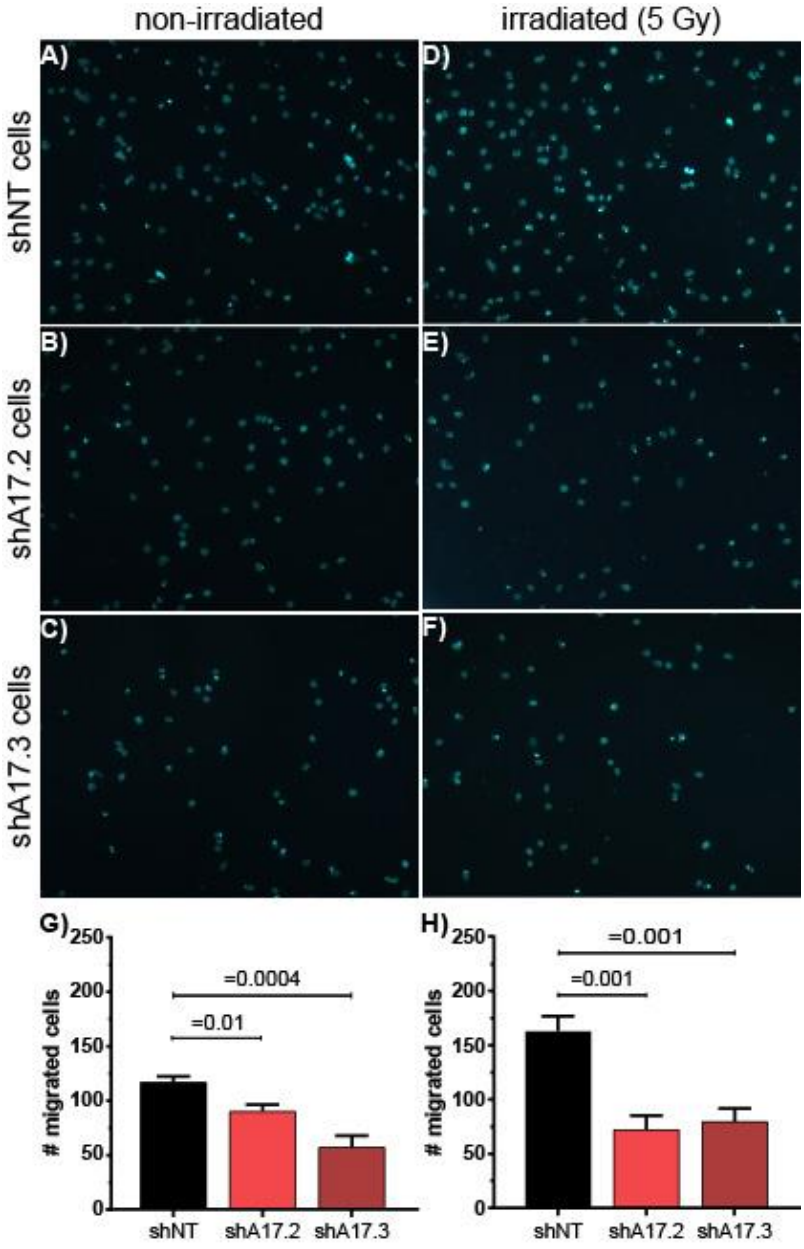

Figure 17. Irradiated A549 shNT cells migrate significantly less towards the secretome of ADAM17-knockdown cells

(**A**)-(**C**) Irradiated shNT A549 cells could migrate towards secretome of non-irradiated control (A) or ADAM17-knockdown cells (B+C). (**D**)-(**F**) Irradiated shNT A549 cells could migrate towards secretome of irradiated control (D) or ADAM17-knockdown cells (E+F). (**G**)+(**H**) For each condition, two separate membranes (n=2) were analyzed and combined data is represented in the bar graph. (G) Data obtained from migration towards non-irradiated secretome (A-C). (H) Data obtained from migration towards irradiated secretome (D-E). Data displayed as mean ± SEM and analyzed with student t test. Significance level $p<0.05$

4 DISCUSSION

Cancer is a leading cause of death worldwide.[55] Albeit being discovered over a century ago, radiotherapy is still a mainstay of current cancer therapy. Ionizing radiation is used as a standard treatment in most patients with solid tumors, either alone or in combination with chemotherapy, targeted therapy or more recently with immunotherapy. Biological factors that can influence treatment outcome include: 1) intrinsic radioresistance of cancer cells, 2) repopulation capacity of surviving cancer cells and 3) degree of hypoxia in the tissue environment. [46]
Over the years, traditional RT has evolved. Newer technologies and combination with other treatment modalities have increased progression free survival and tumor control. Nonetheless, radiation therapy results in unwanted side-effects that could potentially harm the patient and therefore make the therapy unfavorable. Understanding the molecular mechanisms upon IR are therefore of utmost importance.
Ionizing radiation aims to cause DNA damage exceeding the repair capacity of cancer cells and this cytotoxic effect on DNA level has been widely studied. [11, 12, 45] The effect of IR on the tumor microenvironment (TME) and how this can potentially influence treatment outcome is still under investigation. IR induces the secretion of various soluble factors which take part in communication between cells and the TME. This signaling is critical for tissue homeostasis and tumor growth.[16] Deciphering the role of this irradiation-regulated serum proteome, in particular when related to a specific sheddase of interest, as discussed in this project, will help to pave the way towards a combined treatment modality. [9, 18]

ADAM17 is a membrane-associated metalloprotease and actively engaged in proteolytic shedding of membrane-bound proteins. ADAM17 is one of the matrix metalloproteases shown to be upregulated in response to IR and its overexpression is associated with aggressive tumor progression and poor prognoses. [32, 43, 48] The quantitative large scale secretome analysis of Sharma et al showed that ADAM17 sheds multiple key oncogenic factors and that the shedding of these factors is increased following irradiation. Further, they identified ADAM17 as an important resistance mediator in response to IR. By means of these results, ADAM17 is involved in intra- and intercellular mechanism orchestrating radiation resistance, making it an interesting target for the combined treatment with ionizing radiation.

In this study, we show the radiosensitizing effect of ADAM17-downregulation using an inducible shRNA system. Additionally, we provide first hints that ADAM17 is actively involved in intercellular communication, potentially being an important mediator of tumor progression. Our results support the rational of combining IR with ADAM17 inhibitors because of the enzymes active contribution to radioresistance and tumor promoting intercellular communication.

4.1 CHARACTERIZATION OF ADAM17-KNOCKDOWN CELLS

To study the underlying molecular mechanism of how ADAM17 sensitizes towards IR, we generated NSCLC A549 and H358 cell lines containing a doxycycline-inducible expression of a shRNA targeting ADAM17 or a nontargeting shRNA. To visually distinguish the cells, for example by flow cytometry or immunofluorescence, the

vectors with the ADAM17-directed shRNAs contain an RFP tag, whereas the vector with the non-targeting shRNA is supplemented with a GFP tag. A population of A549 cells showed approximately 90% of GFP or RFP positive cells (Figure 10). Each population showed approximately 10% negative cells, resulting either from cells surviving the puromycin selection without insertion of the plasmid because they developed intrinsic resistance or from cells whose expression of GFP or RFP respectively is hampered, resulting in very low or absent expression. To determine if the RFP or GFP negative cells also integrated the plasmid, we could have stained the cell populations with a conjugated-antibody against ADAM17 and gate for the ADAM17-expression on flow cytometry. Additionally, we could have sorted the cells via fluorescent-activated cell sorting (FACS) for the RFP or GFP positive cells, respectively, in order to obtain a population of cells with 100% expression of the respective fluorescent protein.

We showed that the protein levels and activity of ADAM17 decrease prominently in Dox-induced A549 and H358 cells harboring the shADAM17 whereas the shNT did not affect ADAM17 protein levels or activity (Figure 11). Without the addition of DOX, there was no change in protein level, which indicates that the system is not active without doxycycline induction and of low leakage.
Additionally, A549 and H358 shNT cells showed an increase in ADAM17 activity in a time dependent manner following IR, whereas in the A549 and H358 shADAM17 cells this increase was completely abrogated (Figure 12). The ADAM17-directed shRNA diminishes therefore not only the basal level of ADAM17, but also the radiation-dependent increase of ADAM17.

Amphiregulin has been reported as a direct substrate of ADAM17. [41] In order to prove the specificity of the shADAM17 system, we measured the amphiregulin concentration in the supernatant of our shRNA containing cell lines. In line with the reduction of ADAM17 enzyme activity, Amphiregulin was also reduced in the supernatant of shADAM17 cell lines compared to the shNT cell lines (Figure 13A). Interestingly, IR resulted in increased Amphiregulin shedding not only in shNT cells, but also in the shADAM17 cells (Figure 13B), although we did not observe an increase in ADAM17 activity in the shADAM17 cells. This might indicate that upon irradiation, Amphiregulin might be cleaved independently from ADAM17.

Together these results show the efficacy of the Tet-On system, as well as the specificity of shedding events mediated by ADAM17. We are therefore confident to consider these cells as ADAM17-knockdown cells and their secretome as “ADAM17-ligands scarce” and will continue using these genetically engineered cell lines.

4.2 RADIOSENSITIZING EFFECT OF ADAM17 DEPLETION

To cope with IR-induced cellular damage, tumor cells increase the proliferation in the surviving fraction and upregulate pro- survival signals, many of which are mediated by the IR-induced increased ADAM17 activity.[56] In theory, a cell lacking ADAM17 enzyme activity is less capable to cope with IR-induced cellular damage and is therefore more vulnerable to IR. Here we showed that an ADAM17-knockdown A459 cell population had a reduced baseline proliferative activity compared to a control cell population. Treatment with IR further decreased the overall proliferative activity of the

A549 ADAM17-knockdown cell population compared to control cells (Figure 14A). Interestingly, in the H358 cell line, the shADAM17 cell population had the same baseline proliferative activity as the shNT cell population, however upon irradiation a decrease of proliferative activity in shADAM17 cell population compared to a shNT cell population was also observed (Figure 14B). With the AlamarBlue assay we measure the overall proliferative activity of a cell population rather than the proliferative activity of an individual cell. It would therefore be interesting to assess if the decreased proliferative activity in ADAM17-knowdown cells is due to intrinsic mechanisms inhibiting the proliferation of individual cells or if it is a results of increased cell death in the ADAM17-knockdown cell populations resulting in fewer cells that can reduce AlamarBlue. Sharma et al. described another reason for the reduced proliferative activity, namely that upon treatment with IR, ADAM17 knockdown cells go into a senescent state. [41] Concomitantly with the increased radiosensitivity of A549 shADAM17 cells as measured by the proliferative activity, the radiation-dose dependent clonogenicity also decreases compared to control cells (Figure 14C).
From these results we conclude that ADAM17-depletion has a radiosensitizing effect on tumor cells. This synergistic effect on proliferative activity and clonogenic survival of irradiated ADAM17-knockdown cells *in vitro* might be the result of missing autocrine downstream receptor activation. ADAM17 is responsible for the cleavage of multiple ligands that can bind to receptors to activate various downstream signaling pathway - if the ligands are not cleaved, the corresponding growth promoting receptors will not be activated.
Downstream receptor activation (e.g. EGFR phosphorylation status) can be used as surrogate marker to probe for autocrine receptor activation in response to IR. Sharma et al. performed experiments in which they analyzed EGFR phosphorylation over time after IR in control and siRNA-mediated ADAM17-knockdown cells. They saw enhanced EGFR phosphorylation in control cells compared to ADAM17-knockdown cells, and interestingly, also in cells that were subject to conditioned media derived from irradiated control or knockdown cells (siRNA). These results, together with the results from my master thesis, point to an ADAM17-regulated autocrine growth factor loop underlying the radioresistance of tumor cells.

4.3 ADAM17 IS INVOLVED IN INTERCELLULAR SIGNALING PATHWAYS

Besides the ADAM17 dependent autocrine signaling, we were also interested in the paracrine signaling. Therefore, the relevance of the overall ADAM17-dependent secretome to act in a paracrine way was investigated. More precisely, cellular migration, as one component of the paracrine signaling network, was analyzed.

In a first experiment, we showed that A549 ADAM17-knockdown cells migrated significantly less than the shNT cells towards the same attractant, pointing to an ADAM17-dependent autocrine signaling network affecting migration capability (Figure 15).

Next, we tested whether ADAM17-cleaved factors influence the migration capability of surrounding cells.
Here we showed in several experimental settings and to the best of our knowledge for the first time, that migration of A549 shNT cells is significantly reduced when exposed

to a secretome derived from non-irradiated or irradiated ADAM17-knockdown cells as compared to a secretome of non-irradiated or irradiated ADAM17-proficient cells (Figure 16 and 17). These results support the notion that ADAM17-cleaved factors have a paracrine influence on cellular migration.

Irradiation influences cellular migration, although it is not clear whether it enhances or decreases cell migration *in vitro*. For the lung cancer cell line A549, some studies showed increased migration following IR [57, 58] while others showed decreased migration [59, 60]. We reported a slight decrease in migration of irradiated cells compared to non-irradiated cells exposed to a non-irradiated secretome coming from shNT cells (mean of 150 cells/field vs. mean of 117). Surprisingly, when the cells migrated towards a ligand-enriched secretome of shNT cells (generated by irradiation of secretome producing cells), we saw the opposite, namely a slightly higher migration of the irradiated cells compared to the non-irradiated cells. (mean of 163 vs. mean of 148). In other words, the ligand-enriched secretome had no effect on the migration of non-irradiated cells (mean of 150 towards secretome coming from non-irradiated cells vs. mean of 148 towards secretome coming from irradiated cells, $p>0.05$), but it surprisingly increased the migration of irradiated cells (mean of 117 vs. mean of 163, $p=0.013$). The high complexity behind cellular migration as well as the low reproducibility of the transwell migration assay (resulting in high variance), make it difficult to draw any conclusions from the observed results. Nonetheless, we will use these preliminary results to further investigate the migration behavior of A549 in response to IR and towards a dose- and ADAM17-dependent secretome. Additionally, we will test the invasion capabilities of A549 in an *in vitro* invasion assay. Invasion capability can be linked more directly to the multistep process of metastasis, for though migration is a prerequisite for invasion, but not all migrating cells do also invade. [62]

Reflecting on the results from the different transwell migration assays, we propose that ADAM17-cleaved factors are indeed involved in intra- and intercellular communication and migration. The factors either provide an environment more favorable for cancer cells, thus indirectly promoting migration towards the ligand-rich microenvironment, or an ADAM17-cleaved factor can directly promote migration by binding to a receptor whose downstream signaling events activate migration. In so far, the radiation-induced migration and invasion can at least in some part be attributed to ADAM17-mediated regulations. How exactly ionizing radiation influences the ADAM17-mediated signaling processes involved in migration will be addressed in future studies.

Understanding the molecular mechanism behind migration and invasion will help to better comprehend the multi-step process of metastasis. Aggressiveness of tumors increases once they have acquired the capability to invade and metastasize to distant tissues and patients with metastasized tumors are faced with poorer prognosis. With its potential role in intercellular communication affecting migration, ADAM17 might represent a critical target not only for radiosensitization but also for reducing (IR-induced) migration and invasion, altogether improving treatment outcome and patient survival.

4.4 OUTLOOK

Our results support the rational for a combined treatment modality with a potent ADAM17-inhibitor. By inhibiting this highly active protease, NSCLC cells are sensitized towards IR and treatment outcome can potentially be improved. To strengthen these findings, additional experiments *in vitro*, and *in vivo*, are required.

Since ADAM17 expression levels are increased in many different cancers compared to healthy tissue and correlated with poor prognoses, we will additionally study the functional effects of ADAM17 overexpression in order to gain more insights into the regulation of ADAM17 and its influence on auto- and paracrine signaling. We have therefore designed a lentiviral vector that we will use to transduce NSCLC cell lines. The transduced cell lines should then stably overexpress ADAM17. After characterization of the ADAM17 overexpression on the biochemical and cell biological level, we will test whether overexpression of ADAM17 promotes the survival upon irradiation.

Furthermore, we will continue to study the intercellular communication mediated by ADAM17, because the results obtained during my master thesis clearly showed some involvement of ADAM17-cleaved factors. We will need to identify key ligands of ADAM17, acting in an auto- and/or paracrine way and consequently affecting tumor progression or even regression. A promising new method to screen for novel protein-protein interactions is the BioID method. A biotin ligase is fused to a protein of interest, in our case ADAM17, where it can biotinylate proximal proteins. Biotinylation of proteins allows their selective isolation and identification. [61] With this technique we can mark ADAM17-cleaved substrates and eventually identify relevant factors. This will shed light into the complex signaling network of ADAM17 and help to understand how it affects not only the tumor but also the surrounding tissue.

As a closing remark, I want to say that theories and statements mounted in this thesis are based on preliminary data. To comply with high scientific standard, all experiments should be repeated. I hope my findings shed some light into the ongoing research of ADAM17 and will provide a baseline for future experiments done in this field of research.

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